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Manual of Laboratory Operations

Lipid Research Clinics Program

Volume 1
Lipid and Lipoprotein Analysis

May 1974

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Volume 1
Lipid and Lipoprotein Analysis

National Heart and Lung Institute
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PREFACE

The Lipid Research Clinics Program is a collaborative contract research program funded by the National Heart and Lung Institute, one of the National Institutes of Health (NIH), headquartered in Bethesda, Maryland.

NIH supports research and training programs directed against our Nation's leading health problems and is currently responsible for approximately one-third of the medical research conducted in the United States. Its purposes are to increase scientific knowledge of the body and the diseases that afflict it; to improve methods of prevention, diagnosis, and treatment; and to increase the health research resources and manpower of the Nation. Some of this research is conducted in federal laboratories at Bethesda and elsewhere, but most of it is supported through research grants or contracts awarded to scientists and clinicians at universities, medical schools, hospitals and other research institutions throughout the country.

The special province of the National Heart and Lung Institute is the conduct and support of research and training programs concerned with prevention, diagnosis, and relief of cardiovascular, chronic lung and blood disorders.

This manual is the outgrowth of evaluation and research in many laboratories. It arose out of the need of the Lipid Research Clinics Program to have a highly precise and accurate method for blood lipid determination. It is made available now so that the entire scientific community can share in the laboratory techniques and practices that have been validated and standardized by the Lipid Research Clinics Program.

MANUAL OF LABORATORY OPERATIONS
LIPID RESEARCH CLINICS PROGRAM

Vol. I

Lipid and Lipoprotein Analysis

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FOREWORD

Major objectives of the Lipid Research Clinics Program are the determination of the prevalence of primary and secondary hyperlipoproteinemia through studies in selected populations and the Type II Coronary Primary Prevention Trial Study, a trial of the efficacy of cholesterol-lowering in the prevention of coronary heart disease. Each clinic has established a core lipid laboratory to perform the lipid and lipoprotein measurements required by these studies. The need for standard methods leading to highly precise and accurate lipid determinations was recognized from the inception of the program and led to the development of the Lipid Research Clinics Laboratory Methods Manual.

Volume I of this manual is the first of two volumes which detail laboratory procedures used by the Lipid Research Clinics Program and is concerned with the quantitation of human plasma lipids and lipoproteins. The second volume will detail laboratory administrative procedures used in the program's Prevalence Studies and Coronary Primary Prevention Trial and will relate specifically to these studies.

The manual was assembled through the efforts of the Laboratory Methods Committee, whose membership is comprised of representatives of the individual Lipid Research Clinics, the National Heart and Lung Institute, the Center for Disease Control and the Lipid Research Clinics Central Patient Registry. The Committee was ably assisted by the Lipid Research Clinics Pediatrics and Genetics Committee and by various external advisors.

The manual is not considered "finished" but rather represents a continually evolving tool the purpose of which is to allow the accurate, precise and standardized determination of the plasma lipids and lipoproteins. It is primarily a procedural manual but includes occasional brief discussions of various aspects of the procedures where it has been felt necessary to clarify certain points.

The manual necessarily assumes prior knowledge of the lipoproteins and of common laboratory procedures, as well as of the instrumentation. Details of operation of the instruments cover only the procedures used by the Lipid Research Clinics and do not include principles of operation, initial setup or trouble shooting. Users of this manual are urged to be acutely aware of the effects of "cumulative error", which can operate to reduce the acceptability of the lipid analyses. For example, solutions should be accurately prepared, evaporation should be rigorously minimized, and instruments should be operated in a manner which cancels or minimizes the effects of carryover, expansion of standard curves and other possible sources of variation.

Trade names have been used, in certain instances, for the convenience of the reader but this in no way implies the endorsement of those products by the National Institutes of Health, the National Heart and Lung Institute, the Lipid Research Clinics Program or by any of the participants in the program.

Furthermore, the sources of supplies and equipment listed in Appendix I are included for convenience and do not constitute an endorsement. In many cases, alternate sources of equally acceptable materials are available.

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AAI	AutoAnalyzer I
AAII	AutoAnalyzer II
CDC	Center for Disease Control Atlanta, Ga.
CPR	Central Patient Registry Chapel Hill, N.C.
d	density
HDL	High Density Lipoproteins (d 1.063-1.21)
LDL	Low Density Lipoproteins (d 1.006-1.063)
LRC	Lipid Research Clinic
LSL	Lipid Standardization Laboratory
NHLI	National Heart and Lung Institute
NIH	National Institutes of Health Bethesda, Md.
TG	Triglycerides
TM	Trademark
VLDL	Very Low Density Lipoproteins (d$<$1.006)

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1. Obtaining and handling blood specimens.

1.1. Condition of Subjects

Plasma triglyceride levels are markedly influenced by recent intake of fat. For this reason all plasma lipid determinations are done on fasting samples. Patients are instructed to take nothing by mouth other than water for 12-16 hours prior to sampling. In addition, before drawing blood, it is useful to ask the subject about his food intake during the previous 12-16 hours, since this uncovers a small number of subjects who "just forgot" to fast.

Since certain drugs might seriously influence lipid measurements by the methods used, it will be very useful to collect data about medication taken by the patient. Patients may take prescribed medications during the 12-16 hour fasting period if necessary. It should be ascertained whether female subjects are pregnant since blood lipid levels are affected by pregnancy.

1.2. Drawing Blood

It is important to standardize posture (Tan, M.H. et al. N.E.J. Med. 289:416, (1973)). The sitting position seems to be most commonly used. It is difficult in a screening or clinic setting to standardize the length of time that a subject has been in a sitting position prior to venipuncture, since "sitting and waiting" is so common in these circumstances. There may have to be exceptions to the "sitting" rule (e.g. subjects confined to bed). In these cases, posture should be constant for a given subject during a given study.

It is recommended that the venipuncture technician ascertain whether the subject has a bleeding disorder before the blood is drawn.

1.2.1. Sampling Adult Subjects (over 18 years)

The subject should be seated during venipuncture.

Blood should be drawn from an antecubital vein, or failing this, from some other convenient arm vein. Blood may be drawn by a physician, a registered nurse or a licensed medical technologist. Care should be taken to minimize hematoma formation.

A tourniquet is used but should be released prior to withdrawal of the blood sample, if possible, since significant increases in concentration of plasma lipids have been reported as a result of prolonged application of a tourniquet (Koerselman, H.B. et al, J. Atheroscler. Res. 1:85 (1961), Page, I.H. and Moinuddin, M., Circulation 25 651 (1962)). It may be difficult, in some cases, to obtain good flow without continuous application of the tourniquet.

Blood is drawn using the Vacutainer TM* system following the instructions supplied with the system. Vacutainer TM tube stoppers should be lubricated with silicone rather than glycerine to avoid interference with triglyceride determinations. A 1-1/2", 20 gauge needle is placed in the vein, the tourniquet removed, and the required number of Vacutainer tubes, containing solid disodium EDTA, are filled as completely as possible.

Use of solid EDTA as anticoagulant eliminates dilution which would occur with tubes containing a solution of EDTA in saline. Plasma is preferred to serum for lipid determinations because 1) EDTA chelates heavy metals that may promote autooxidation of lipids, 2) there is no need to allow blood to remain at room temperature for coagulation to occur, and 3)

*TM Becton-Dickenson Co., Rutherford, N.J.

it is difficult to recover chylomicrons from lipemic sera.

Where several tubes are to be drawn, the use of non-drip multiple sample needles is recommended. The filled tubes are promptly mixed by inverting eight times. Mixing must be prompt and thorough, but not too vigorous. The tubes are then labeled with appropriate patient identification and placed in wet ice, pending removal of plasma.

1.2.2. Pediatric Subjects (birth to 18 years)

A) Sampling at Birth

1) Following the delivery of the infant, the umbilical cord is clamped 2-3 inches (5-7 cm) from the umbilicus and the cord severed distal to the clamp.

2) Approximately 30-35 ml of cord blood is allowed to drip into two or three EDTA containing Vacutainer™ tubes with constant agitation of the tube to insure mixing of the anticoagulant with the blood. If 30-35 ml is not obtainable, then 15 ml should provide enough plasma (0.6 (average hematocrit of blood) x 15 ml = 9 ml of erythrocytes + 6 ml of plasma) for determination of the plasma total and HDL cholesterol, and triglyceride. The blood should be immediately cooled in wet ice and stored at $4^{o}C$ prior to processing.

3) Precautions

a) Bilirubin

Cord blood bilirubin is usually between 1-2 mg/100 ml of plasma at birth (Davidson, L.T. Amer. J. Dis. Childh. 61:958, (1941)). Although hyperbilirubinemia may interfere with the determination

of cholesterol (Moline, O., Clin. Chem. 15: 521, (1969)), the problem is circumvented by the use of zeolite mixture at the extraction stage (zeolite mixture absorbs bilirubin).

b) Maternal contribution of lipids

Significant amounts of lipids and lipoproteins do not ordinarily cross the placenta (Zee, P., Pediatrics, 39:82, (1967); Sabata, V., Gynaecologica, 163:179, (1967)), but the possibility of maternal contributions to the umbilical cord blood sample is increased by delayed clamping of the cord (Boyd, E.J., Clin. Invest. 14:7, (1935), Dancis, J., Am. J. Obstet. Gyn., 84:1749, (1962)). Consequently, the cord should be clamped quickly and the cord blood collected as soon as possible following birth.

B. Sampling in the First Year of Life

1) Amount of Blood

In the first year of life, the circulating blood volume (CBV) is approximately equal to 8% of the total body weight. It is considered safe to draw approximately 5% of the circulating blood volume. Thus a table may be constructed:

Age (months)	Average Weight (white males, kg.)	CBV* (ml)	5% of CBV (ml)
Birth	3.36	268.8	13.4
1	4.72	377.6	18.9
2	5.44	435.2	21.8
3	6.17	493.6	24.7
4	6.80	544.0	27.2
5	7.17	573.6	28.7

6	7.85	628.0	31.4
7	8.16	652.8	32.6
8	8.48	678.4	33.9
9	8.80	704.0	35.2
10	9.07	725.6	36.3
11	9.39	751.2	37.6
12	9.71	776.8	38.8

*CBV circulating blood volume

At age 6 months, 30-35 ml may be drawn from a child. From 2-6 months, 20-25 ml is a safe amount, with 15 ml for infants less than 2 months of age. These are the recommended maximum amounts. For the purpose of screening, 15 ml should be drawn.

2) Plasma Sampling and Samples

The Pediatrics-Genetics Committee of the LRC project recommends that a physician only should draw blood samples from any child aged one year or less. This procedure should be performed in a hospital or similar setting (e.g. doctor's office). The blood should not be drawn by a venipuncture technician.

3) Technique of Sampling

The area is prepared prior to sampling with an antiseptic swab. Blood may be drawn from the antecubital fossa, dorsum of the hand or the external jugular vein. Blood should NOT be drawn from the femoral vein because of its proximity to the femoral artery and nerve. A sterile Butterfly-23 Infusion Set is recommended to give the maximum maneuverability. Some infants may require a Butterfly Short-25 Pediatric Infusion Set.

4) Diet

a) The kind of milk in the diet (e.g. breast milk, whole cow's, commercial formula brand) should be noted at the time of sampling.

b) The sample should be obtained before the morning feeding following an 8-10 hour fast. If a feeding must occur between midnight and 8 a.m., 4 oz. (120 ml) of a 5% dextrose solution should be provided by the Clinic as a substitute for the night feeding.

C) Sampling at Age 1-18 Years

1) The venipuncture site should be prepared as described previously. Thirty-five ml should be drawn from the antecubital fossa or dorsum of the hand using the same needle as described for adults; or, in smaller children, the Butterfly-23 may be more convenient.

2) The blood may be drawn by a venipuncture technician, but when possible, a physician should draw the blood from children aged one to three years. The sample may be collected in the home, at school, or in the hospital.

3) The period of fasting should be the same as for adults.

1.3. Preparation of Plasma

Blood samples are cooled on wet ice as soon as they are collected. Within 3 hours (and preferably within 1 hour) the tubes are centrifuged at 4°C in a refrigerated centrifuge at 1500 Xg for 30 minutes (45,000 g-min). If a refrigerated centrifuge is not available with-

in 3 hours of collection, the samples may be centrifuged at room temperature within 1 hour of collection, and the plasma stored at 4°C.

Plasma is promptly separated from the cells, using a Pasteur pipet with a rubber bulb. The pipet should be large enough so that the plasma cannot be drawn into the bulb. The white cell layer (buffy coat) is not transferred with plasma.

1.4. Labeling and Storage of Plasma

The plasma is transferred to a clean 20 ml scintillation vial with polyseal cone cap. The total plasma from one blood drawing (one or two Vacutainers™) is mixed and the appropriate label applied to the vial. The vials should not be filled beyond the shoulder (about 20 ml) in order to leave an air space for mixing and expansion.

The caps should be screwed down tightly and the vials mixed well and stored at 4°C in the dark.

Use of the 20 ml scintillation vials facilitates storage of large numbers of samples in flat trays of 100 vials. Color coding, using adhesive paper discs on the caps, may be useful in identifying vials.

1.5. Shipment of Plasma Samples for Lipid and Lipoprotein Determination

Plasma samples on which total cholesterol and triglycerides only are to be determined can be conveniently shipped frozen in dry ice. When it is necessary to ship plasma samples for lipoprotein determination, these samples must not be frozen and the following system may be used. The plasma should be transferred from the Vacutainer™ to a 20 ml scintillation vial. The cap is tightened and secured with tape. The vial should be properly labeled and placed in a styrofoam sheet holder. The tops of the vials are then covered with a plexi-

glass cover which is held in place with a rubber band. Crushed ice is sealed in a plastic food bag and placed in the bottom of an insulated shipping container. The styrofoam holder with vials is then placed on top of the ice. A second bag of ice is placed on top of the samples. The items required are listed in Appendix I.

1.6. Caution in Handling Plasmas

Care must be exercised in handling blood and plasma. Do not use mouth pipets. Avoid any contact with plasma. Cover any cuts or scratches on fingers very carefully before handling plasma.

2. Automated Determination of Plasma Cholesterol and Triglycerides.

Analyses are performed by a two step method involving 1) preparation of an isopropanol extract of plasma and subsequent treatment with zeolite mixture to remove phospholipids, glucose, bilirubin and certain other substances which may interfere with the determinations, and 2) the simultaneous determination of cholesterol and triglycerides by use of the AutoAnalyzer^{TM*} I or II. The colorimetric determination of cholesterol with the AAI depends on the formation of a purple color developed in the presence of a sulfuric-acetic acid mixture containing Fe^{3+}. Colorimetric determination of cholesterol with the AAII depends on the formation of a blue color developed with a sulfuric-acetic acid mixture containing acetic anhydride. Both methods measure a mixture of free cholesterol and cholesterol esters. Both systems measure triglycerides fluorometrically after hydrolysis to free glycerol and subsequent oxidation of the glycerol to formaldehyde followed by coupling with acetylacetone to give the fluorescent product 3,5-diacetyl-4-dihydrolutidine.

2.1. Preparation of Sample Extracts

2.1.1. Equipment and Supplies

A) Sample Dilution Equipment

The MicroMedic^{TM*} Automatic Pipet is used for aliquoting 0.5 ml of either plasma sample, isopropanol standard, 99% isopropanol, or saline, and for dispensing 9.0 ml of 99% isopropanol. Instructions provided with the pump should be carefully followed.

A calibration check, based on accurate weighing of the delivered volume of isopropanol and plasma, should be conducted at two-week intervals. In addition, the

*^{TM}Technicon Instruments Corp., Tarrytown, N.Y.
*^{TM}Micromedic Systems, Inc., Pennsauken, N.J.

AutoAnalyzer response by the method described, for a given plasma, may be compared with the response for the plasma obtained using "A" grade glassware to dispense the plasma and isopropanol.

B) Volumetric Glassware

Class A pipets and volumetric flasks are to be used for the preparation of solutions. Mohr and serological pipets should not be used.

C) Extraction Tubes and Closures

A supply of 16x125 mm screw-cap tubes, with Teflon™* lined screw caps is required. Disposable tubes are available. The use of disposable tubes eliminates chipped tubes which may leak.

D) Adsorbent Dispenser

A metal spoon or glass scoop containing about 2.0 g of zeolite mixture when filled in a standard way (i.e. leveled off) is recommended. This may be used in conjunction with a wide-stem Teflon™ funnel to add 2.0 g of zeolite mixture to each extraction tube. A glass rod may be required to loosen the powder from the sides of the funnel to effect complete transfer to the tube.

E) Centrifuge for Extraction Tubes

A centrifuge equipped with either a fixed or a swinging bucket rotor can be used. If the centrifuge is refrigerated, allow samples to come to room temperature before analyzing.

F) Automated Analytical Equipment

*™Dupont Co., Wilmington, Delaware

Instructions are given for analyses performed with the Technicon AutoAnalyzers™ I and II (secs. 2.2. and 2.3.).

G. Reagents Needed for Preparation of Extracts (see sec. 2.2.1.)

2.1.2. Glassware Washing

Disposable extraction tubes are used. The Teflon™-lined plastic caps are washed in detergent, thoroughly washed in water, rinsed finally with distilled water and dried in an oven at low temperature ($< 45^{\circ}C$).

All glassware used for the determination of triglycerides should be carefully washed free of fluorescent contaminants. Detergents with fluorescent components should be avoided. Glassware is acid washed and rinsed with distilled water. Volumetric glassware should not be dried in hot ovens.

2.1.3. Standards

For purposes of the Lipid Research Clinics project, combined concentrated solutions of pure standard cholesterol and triolein in 99% isopropanol will be supplied as needed by the Lipid Standardization Laboratory, CDC, Atlanta, Ga., at the following concentrations (cholesterol, mg/100 ml/triolein, mg/100 ml): S_1 - 100/50; S_2 - 200/100; S_3 - 300/200; S_4 - 400/300; S_5 - 50/0. These standards must be kept in a refrigerator in tightly closed dark bottles.

The S_5 (50 mg/100 ml cholesterol) standard is specifically for use when analyzing plasma fractions which have low concentrations of cholesterol (< 100 mg/100 ml).

The average LRC Laboratory temperature is considered to

be about 23°C. Thus all liquids should be at or near this standard temperature during volumetric operations. Plasma samples (unknown or control pools) should also be brought to this temperature before aliquoting. Standard solutions in isopropanol are aliquoted with the MicroMedic™ pump (1 ml syringe set at 50%, 5 ml syringe set at 90%). Standard solution (S_1, S_2, S_3, S_4, or S_5), 0.15 M saline and 99% isopropanol is dispensed into an extraction tube with the following pump sequence:

(a) 0.5 ml of concentrated standard

(b) 4.50 ml of 99% isopropanol

(c) 0.5 ml 0.15 M saline

(d) 4.50 ml of 99% isopropanol

For reagent blank, use the same sequence as for standard, but in (a) substitute 99% isopropanol for standard. The resulting solution is comparable in composition to that of plasma extracts.

Extracts made from standard solutions are then treated with zeolite mixture (see sec. 2.1.5.).

2.1.4. Quality Control Samples

For the purposes of the Lipid Research Clinics project the quality control samples (frozen sera with known levels of triglycerides and cholesterol) are supplied by the Lipid Standardization Laboratory, CDC, Atlanta, Ga. The samples should be kept at -20° to -60°C in the upright position until used.

Quality control samples with known concentrations of cholesterol and triglycerides are included with each day's

run. Two extracts are prepared for samples at the lower concentration and two for samples at the higher concentration for a total of 4 extracts. Each of the extracts is run in duplicate, so that four determinations of the lower control serum, and four of the higher control serum, are included with each day's run, regardless of the total number of trays included in that run. This system is explained more fully in section 3.

For each day's work, the quality control samples should be removed from the freezer, allowed to thaw, and to attain room temperature. The vials are then gently swirled and inverted several times in order to rinse the cap and to mix the sample. The sample should stand for a few minutes to allow the serum to drain from the cap.

2.1.5. Extraction and Treatment of Plasma Samples, Standards and Quality Control Samples

A) Extraction of Plasma and Quality Control Samples

Unknown plasmas and control pool sera are delivered into extraction tubes and extracted with isopropanol. Plasma and isopropanol should be at approximately 23°C. The MicroMedic™ pump (1 ml syringe set at 50%, 5 ml syringe set at 90%) is used with the following pump sequence:

(a) 0.5 ml of plasma	(a) 0.5 ml 99% isopropanol
(b) 4.5 ml 99% isopropanol	(b) 4.5 ml 99% isopropanol
or	
(c) 0.5 ml 99% isopropanol	(c) 0.5 ml plasma
(d) 4.5 ml 99% isopropanol	(d) 4.5 ml 99% isopropanol

Each tube is capped and immediately vortexed for 10 seconds. A fine dispersion of protein precipitate results.

B) Treatment of Extracts with Zeolite Mixture

The following instructions apply to standards, (sec. 2.1.3.), and extracts of both patient and quality control samples. The cap is removed from the first tube, 2.0 g of zeolite mixture added, the cap replaced and the tube is vortexed for 10 seconds. Subsequent tubes are similarly treated, and allowed to stand for at least 30 minutes after vortexing. After this time each tube is vortexed again for 10 seconds. It is convenient for an operator to work with two vortex mixers simultaneously. During all vortexing, keep the tube contents away from the cap to avoid transfer of zeolite to sample cups later.

The tubes are centrifuged at 1500 x g for 15 minutes at room temperature, to firmly pack the zeolite. If a refrigerated centrifuge is used, samples should subsequently be allowed to return to room temperature before analysis. The supernatant extract may be decanted directly into sample cups. If analysis is delayed overnight, the extracts should be decanted into clean, labeled extraction tubes. The tubes are firmly capped, and stored in the refrigerator for no more than 3 or 4 days.

2.2. Operation of the AutoAnalyzer I

2.2.1. Reagents

A) Isopropanol

Isopropanol (99%) with minimum fluorescence must be employed.

New lots of isopropanol may be tested as follows:

Pump reagents through the AA/I, placing the triglyceride probe in distilled water. Switch the triglyceride probe to 99% isopropanol until a steady baseline is obtained. An increase in baseline fluorescence (isopropanol versus pure water) in excess of 5 mg/100 ml triglyceride equivalence indicates unacceptable isopropanol. Excess fluorescence can usually be removed from isopropanol by distillation or distillation over sodium borohydride. Since isopropanol is mildly hygroscopic, vessels containing this solvent should not be left exposed to air.

B) Zeolite Mixture

Grind zeolite to a powder in a blender at high speed; place in shallow pan or tray and heat overnight at 100°C. Cool and place 200 g in a wide mouth jar. Grind 10 g of copper sulfate ($CuSO_4 \cdot 5H_2O$) in a mortar and pestle. Mix the ground copper sulfate, 20 g of Lloyd's Reagent, 20 g calcium hydroxide, and the zeolite. The jar should be stoppered tightly, and stored in a desiccator over anhydrous calcium sulfate until used. A prepared anhydrous zeolite mixture is available commercially and may be reactivated by heating at 110°C overnight.

C) Cholesterol Color Reagent

Prepared fresh daily.

825 mg of anhydrous reagent grade ferric chloride is added to two liters of reagent grade glacial acetic acid in a four-liter erlenmeyer flask. The ferric chloride dissolves readily at room temperature. Cool the flask for 20 minutes in an ice bath and then add slowly one liter

of reagent grade sulfuric acid. The solution is then mixed for fifteen minutes using a magnetic stirrer and transferred to glass-stoppered dark bottles.

During these procedures exposure to atmospheric moisture should be minimized.

NOTE: THIS REAGENT SHOULD BE PREPARED IN A FLASK WHICH IS COOLED IN AN ICE BATH OF SUFFICIENT SIZE TO CONTAIN THE ACID IN THE EVENT THE FLASK BREAKS. PROTECTIVE CLOTHING AND GOGGLES MUST BE WORN. THESE PROCEDURES SHOULD BE CARRIED OUT IN A FUME HOOD.

D) Alcoholic Potassium Hydroxide

Alcoholic potassium hydroxide is commercially available, however a more stable TG baseline is obtained when this reagent is freshly made.

Dissolve 5 g of reagent grade solid potassium hydroxide in 250 ml of distilled water and bring to one liter with isopropanol (99%). Mix well. Make fresh for each day's run and keep in an amber bottle during use.

NOTE: If base reagent is to be mixed "on-line" (see sec. 2.2.2. below) the following solutions should be prepared instead of base reagent:

1) 20 g solid potassium hydroxide is dissolved and brought to 1 l with distilled water.
2) 85% isopropanol (v/v). Add 150 ml distilled water to 850 ml 99% isopropanol.

E) Sodium Periodate Reagent

This reagent should be prepared in a fume hood. Place 500 ml of distilled water in a one liter volumetric flask. Slowly add 115 ml glacial acetic acid; swirl until mixed, then add 5.4 g of sodium periodate. After it is completely dissolved, dilute to volume with distilled water. The commercially available solution contains 0.54% sodium periodate in 2.01 N acetic acid. If commercial reagent is used specify its shipment in glass containers.

F) Ammonium Acetate, 2 M

Commercially available or dissolve 154 g reagent grade ammonium acetate and bring to one liter with distilled water. The solution should be adjusted to pH 6.0 daily before use by addition of glacial acetic acid.

G) Acetylacetone Reagent (Prepared Fresh Daily)

7.5 ml 2,4-pentanedione (acetylacetone) is dissolved in 25 ml isopropanol in a one liter volumetric flask. After complete solution, dilute to volume with 2M ammonium acetate (pH 6.0). Transfer to an amber bottle.

This reagent should stand for thirty minutes to one hour at room temperature before use since a more stable triglyceride baseline has been observed under these conditions. It should be kept in a dark bottle during the analytical run.

H) Distilled Water

Quality of water is quite critical. All-glass distillation from a good source of deionized water is

recommended.

I) Quality of Reagents

In spite of the preceding precautions a poor quality reagent may be encountered. Before each run, reagents are drawn through the lines until a steady baseline is registered for both recorder pens. Any large deviation from the instrument settings of the previous day suggests a poor quality reagent, or deterioration of pump or other tubing. If attention to the tubing does not improve matters, the offending reagent should be systematically sought and replaced before proceeding with the run.

2.2.2. Preparation for the Analysis

A) All reagents and extracts are brought to room temperature. Check quantities of all reagents.

B) Check the AutoAnalyzer™ I equipment (see flow sheet and details, Fig. 1 and Table 1).

1) Replace the two Acidflex™* base reagent pump tubes every day or every second day of operation, unless on line mixing is used (see sec. 2.2.2.E.).

2) Replace the three color reagent pump tubes every day or every second day.

3) Replace the two Solvaflex™* sample pump tubes every second day.

4) Replace all other pump tubes once each week.

5) Check all other lines for deterioration and replace when necessary.

*™Technicon Corp. Tarrytown, New York

6) Replace any pump tubes more frequently than stated above if significant flattening is observed.

7) Check that connections between modules are kept as short as possible.

C) Start pumping triglyceride reagents and continue until a steady baseline is obtained. Then adjust triglyceride baseline to 5% of full scale deflection. Run one or more S_4 high standards through the triglyceride sample line and adjust the fluorometer span to a convenient position on the chart paper (approximately 75% of full scale deflection). Improvement of triglyceride baseline may be obtained by use of a one-kilowatt voltage regulator with the fluorometer.

D) Start pumping the cholesterol color reagent about ten minutes before starting the analysis. Adjust the cholesterol baseline to approximately 97% transmission.

Check the baseline and standard calibration settings for both the fluorometer and colorimeter, record and compare with the previous day's readings.

E) Improved triglyceride results have been obtained by modification of the system to allow "on-line" mixing of the potassium hydroxide and isopropanol solutions (Noble, R.P. and Campbell, F.M., Clin. Chem. 16:166 (1970)), thereby minimizing deterioration of the solution during storage and eliminating the Acidflex™ tubes.

Remove the two base reagent Acidflex™ tubes (positions 21 and 22, Fig. 1). Replace these with a standard (Tygon) 0.035 tube in position 21 and a Solvaflex 0.081 tube in

position 22. Use the same glass connection as before. Connect the tubing to the glass-connection facing the sampler. Each tubing now goes to a different reagent (Standard 0.035 tube is used for the 2% potassium hydroxide solution and the Solvaflex™ 0.081 tube is used for the 85% isopropanol solution (sec. 2.2.1.D.)). This combination gives the same proportions of potassium hydroxide/isopropanol/water as that specified in the reference cited above, but gives a reagent volume which is more suitable for the present version of the AAI.

2.2.3. Analytical Run

A) The run is conducted using water in the cholesterol and triglyceride wash receptacles. A forty per hour 2:1 sample/wash cam is used on the Sampler IV.

B) Use of Standards and Controls

Each day's work will include standards and quality control samples. The standards are four concentrations, S_1, S_2, S_3 and S_4 (sec. 2.1.3.). When low cholesterol values are anticipated S_5 may be substituted for S_4. Samples from two quality control pools contain normal and elevated cholesterol and triglyceride levels. Samples from two other pools (approximately 50 mg cholesterol/100 ml serum) will be used when ultracentrifugal top fractions ($d < 1.006$) and HDL samples are assayed (sec. 3 and 4).

An ascending (S_1, S_2, S_3, S_4)series of standards is used on each tray, immediately followed by a descending

(S_4, S_3, S_2, S_1) series. A similar array of standards is also run following the final sample analyzed that day. In addition, four normal and four high quality control samples are run each day. (The number of analyses of a given control pool during a day's operation is kept constant in order to facilitate construction of quality control charts. Four analyses are considered most suitable for general purposes). The arrangement of standards and samples on trays is illustrated in Table 2. Thus four low and four high quality control samples are used regardless of the number of samples analyzed that day.

The suggested distribution of quality control samples is shown in Table 3.

C) Zeolite-treated extracts of standards, quality control samples and unknown plasmas are loaded onto trays (one tray at a time) as indicated in Tables 2 and 3. The plastic cover is placed over the forty 5 ml sample cups.

Allow the analysis to proceed, and identify each peak on the triglyceride and the cholesterol recorder tracings.

Check the linearity of the cholesterol (semilog) and triglyceride (linear) standards by plotting on graph paper.

Be sure the sample probes are not closer than 2 mm from the bottom of the cups in order to minimize the chance of aspiration of any zeolite particles transferred to the cups.

2.2.4. Triglyceride Blanks

A) Triglyceride blanks on the great majority of fresh plasma samples are usually less than 5 mg/100 ml equivalence and are frequently zero. Occasionally, plasma samples with triglyceride levels above 300 mg/100 ml have a significant blank due to certain drugs or other causes. Also blank subtraction has been found essential for the high and low quality control samples and for other samples supplied by the Lipid Standardization Laboratory so that AutoAnalyzer™ values to approximate target values for triglyceride content.

The blank determination described here is not a true blank, but only an approach to the problem of extraneous fluorescent substances in the extracts.

B) Triglyceride blanks are run on the following:

1) All plasma samples with off-scale triglyceride readings (i.e., values greater than 300 mg/100 ml on the AutoAnalyzer™) when they are rerun following dilution of plasma and reextraction (see sec. 2.2.5.).

2) All quality control samples supplied by the Lipid Standardization Laboratory for triglyceride analysis.

(Triglyceride blanks are not run on standards.)

C) All blanks are conveniently run together, at the end of a day's run. When blank determination must be postponed, sufficient extract is stored at 4°C for this purpose.

D) Blanks are determined by diverting the complete reaction mixture from the 50°C hydrolysis heating bath. A permanent bypass consists of two three-way stopcocks (Appendix I) connected by the minimum length of 0.090 Solva-

flex™ tubing. The exact location of the bypass is indicated on Figure 1 by a dashed line.

E) Determine triglyceride blanks as follows:

1) Adjust the position of the stopcocks to bypass the heating bath.

2) Pump reagents for ten minutes to restablish baseline.

3) Run all appropriate sample extracts.

4) Sample extract readings are converted to equivalent triglyceride concentration by referring to the most recently run standard curve.

2.2.5. Treatment of Samples Giving Off-Scale Instrument Response

When a sample gives an instrument response beyond the upper limit of the range of standards, ($\rangle$ 400 mg/100 ml cholesterol or $\rangle$ 300 mg/100 ml triglycerides) the analysis is repeated. The original plasma should be carefully diluted with 0.15 M saline to an extent which must be estimated from the instrument response. The diluted plasma (0.5 ml) is then extracted and treated with zeolite mixture as described in sec. 2.1.5.

Care must be taken to multiply the concentrations obtained by the appropriate factor to allow for the dilution. Blanks are subtracted from the values obtained on the diluted plasma, after which the dilution factor is applied. When the value of the blank itself is to be reported, it will be necessary to multiply the blank observed on the diluted-plasma extract by the dilution factor to obtain the blank which applies to the undiluted sample.

2.2.6. Shutdown Procedure

At the end of the run all reagent and sample lines are placed in a large vessel of water and pumping is continued until several hundred milliliters of water have been pumped through. Note that when the lines are washed the heating bath coil that was bypassed must be washed also by returning the stopcocks to their normal position. If this is not done the coil may become blocked. The lines are then removed from the water and air-dried by pumping air for a further thirty minutes. The pump is then switched off and the platen and cover removed from the pump. All instrument switches are turned off. Both heating baths remain permanently on.

2.2.7. Computation of Results

The method described (which in effect plots the average of four points for each standard including the isopropanol zero) takes into account carryover, drift of baseline, and any change in response of standards from tray to tray.

Standard curves for both triglyceride (linear) and cholesterol (semi-log) should be linear. Four values are then read off for each standard (S_1, S_2, S_3, S_4): the ascending and descending values for standards both at the beginning of a given tray and at the beginning of the next tray. In addition four values are obtained for the 95% isopropanol zero, using the values for the second and third isopropanol cups both before and after a given tray (Table 2). The mean of the individual values for each standard and the isopropanol is calculated and used to plot the standard curve for that tray.

The final set of values for ascending and descending

standards, and for 95% isopropanol cups, are used in constructing the curve for samples on the last tray of the day's run. Results for all samples on a given tray are then calculated from the standard curve constructed for that tray. Alternatively results can be calculated by computer using a program which accepts the raw instrument readings.

2.3. Operation of the AutoAnalyzer^{TM}II

2.3.1. Reagents

A) Lieberman-Burchard Reagent. (Cholesterol Color Reagent):

Extra care must be taken while preparing this reagent due to the extreme corrosiveness and volatility of the reagents used. PROTECTIVE EYE GOGGLES SHOULD BE WORN AT ALL TIMES WHILE PREPARING THIS REAGENT IN A FUME HOOD WITH ADEQUATE DRAFT.

Place 600 ml of acetic anhydride in a two liter glass stoppered erlenmeyer flask containing a magnetic stirring bar. Slowly add 300 ml of glacial acetic acid and stir on magnetic stirrer until completely mixed (approximately 5-7 minutes). Transfer the flask to an ice bucket large enough to accommodate the bottom of the flask. Add 95% ethanol to the ice bucket around the flask to slightly less than the level of the liquid inside the flask. Add several small pieces of dry ice to the alcohol for cooling, place the ice bucket onto a stirring plate and stir slowly. Mount a burette containing 100 ml of cold conentrated sulfuric acid above the flask so that the tip is inside and just beneath the neck of the flask. When the

first ice cyrstals appear, start dripping the sulfuric acid not so fast as to splash but not so slowly as to let many more ice crystals form. If vertical space is a problem, a separatory funnel may be substituted for the burette. After the acid has been added, remove the flask from the ice bucket, dry and replace on the stirrer for an additional ten minutes. Transfer the reagent to a glass stoppered amber bottle, seal with parafilm and store at 4°C until used. The solution should be clear and almost colorless. The reagent can be safely stored for one week before use.

B) 0.8 M Potassium Hydroxide

Place 500 ml of distilled water in a one liter volumetric flask and add 45 g of potassium hydroxide; shake until completely dissolved. Dilute to volume with distilled water. This reagent is also commercially available.

C) Sodium Periodate Reagent

This reagent should be prepared in a fume hood. Place 500 ml of distilled water in a one liter volumetric flask. Slowly add 115 ml glacial acetic acid; swirl until mixed, then add 5.4 g of sodium periodate. After it is completely dissolved, dilute to volume with distilled water. The commercially available solution contains 0.54% sodium periodate in 2.01 N acetic acid. If commercial reagent is used specify its shipment in glass containers.

D) 2.0 M Ammonium Acetate

Commercially available or dissolve 154 g reagent grade ammonium acetate and bring to one liter with dis-

tilled water. The solution should be adjusted to pH 6.0 daily before use by addition of glacial acetic acid.

E) Acetylacetone Reagent

Prepared fresh daily.

7.5 ml 2,4-pentanedione (acetylacetone) is dissolved in 25 ml isopropanol in a one liter volumetric flask. After complete solution, dilute to volume with 2M ammonium acetate (pH 6.0). Transfer to an amber bottle.

This reagent should stand for thirty minutes to one hour at room temperature before use, since a more stable triglyceride baseline has been observed under these conditions. It should be kept in an amber bottle during the analytical run.

F) Zeolite Mixture

Grind zeolite to a powder in a blender at high speed; place in shallow pan or tray and heat overnight at 100°C. Cool and place 200 g in a wide mouth jar. Grind 10 g of copper sulfate ($CuSO_4 5H_2O$) in a mortar and pestle. Mix the ground copper sulfate, 200 g of Lloyd Reagent, 20 g calcium hydroxide, and the zeolite. The jar should be stoppered tightly and stored in a desiccator over anhydrous calcium sulfate until used. A prepared anhydrous zeolite mixture is available commercially and may be reactivated by heating at 110°C overnight.

G) General

All chemicals should be of reagent grade and the isopropanol should be free of fluorescence by use-testing

on the AutoAnalyzer.™ Any large deviations of baseline or instrument settings suggest poor quality reagent(s) or deterioration of pump or other tubing. If attention to the tubing does not improve matters, the offending reagent should be systematically sought and replaced before proceeding with the run.

2.3.3. Preparation for Analysis

To Be Done Daily: (See Figure 2 and Table 4)

A) Change color reagent pump tubing and pull through line.

B) Turn on Haake bath. The distilled water level should be about one-half inch from top. The temperature of the bath should be 60°C. Bath must be connected to an independent electrical circuit.

C) Turn on colorimeter and fluoronephelometer at least thirty minutes before use.

D) Pump air through all manifold tubes except those which supply the wash reservoirs. Pump distilled water to the cholesterol wash reservoir and 60% isopropanol to the triglyceride reservoir. Continue pumping for thirty minutes.

E) Start pumping color reagent and lower probe into cholesterol wash receptable.

F) Start pumping 80% isopropanol and 0.8M potassium hydroxide. The air gun should release one bubble every two seconds.

G) When the mixture of 80% isopropanol and 0.8M potassium hydroxide emerges from the hydrolysis bath of the triglyceride cartridge, start pumping sodium periodate and

acetylacetone reagent. Lower the triglyceride probe into the triglyceride wash receptacle.

H) Turn on Printer Module (red "Power On") and then turn on Channels A & B. Set range for cholesterol at 500 and for triglyceride at 400 using the "Range" control.

I) Turn recorder system switch from "Off" (middle position) to "1 + 2" position. Turn Chart Drive on.

J) Adjust the green pen (cholesterol) with colorimeter zero and full scale adjustment controls. Adjust the visual display to agree with the position of the recorder pen at both 0 and 500. Turn the "Function" switch to "Normal" or "Damp 1". (Never use Damp 2 since this position excessively reduces the sensitivity of the instrument).

K) 1) While triglyceride reagents are equilibrating, turn fluoronephelometer "Sample" aperture to "C". Adjust "Baseline" and "Standard" calibration potentiometers to 0.00. Set "Function" switch to "Reference" position and set "Reference" aperture to "1". The red pen should read $15^{+}_{-}1$ on the recorder. If necessary, set reference energy with light pipe wheel by removing the metal plug immediately to the left of the reference aperture wheel inserting a small screwdriver and turning the knurled setting screw one way or the other (see manufacturers instructions). It may be necessary to increase the "Reference" aperture in order to make this adjustment. Return "Function" switch to "No Damp" position. Pen should trace zero. If not, adjust zero with "Rec. Zero" screw on lower

left of fluoronephelometer.

2) After reagents have been pumping for at least ten minutes, turn "Sample" aperture to "2", and "Standard" calibration potentiometer to 3.0. Red recorder pen should read approximately 17.5. If necessary, adjust by increasing or decreasing "Sample" aperture.

3) Adjust position of flow cell for maximum sensitivity if necessary. (i.e., "peak" the flow cell. See manufacturers instructions).

4) Adjust reagent baseline to zero on recorder using "Baseline" potentiometer. The reading on "Baseline" potentiometer scale should be no higher than 5.5 and should be recorded daily.

L) Turn on the sampler and aspirate four cups of zeolite treated isopropanol through both cholesterol and triglyceride probes.

1) When the isopropanol emerges, adjust green (cholesterol) pen to read zero on the recorder using the colorimeter baseline control. Adjust visual display to zero if necessary with the "Zero" control on the cholesterol channel of the digital printer.

2) When the isopropanol emerges, adjust baseline of red (triglyceride) pen to read zero on the recorder using fluoronephelometer "Baseline" potentiometer. Adjust the visual display to zero if necessary with the "Zero" control on the triglyceride channel of the digital printer.

3) These baseline settings are readjusted only at the

beginning of each tray.

4) Set the sample identification on digital printer to "001".

M) The sequence of analyses is set up in a written program before beginning the day's run. The eight quality control samples are distributed among all unknown sera run that day, according to the sequences given in Tables 5 and 6. The linearity of the cholesterol and triglyceride standard curves is established at the beginning of each day's run. The reading of the S_3 standard on the recorder is readjusted at the beginning of each tray.

1) Fill the sample cups to just above the area where the cup widens (2 - 3 ml).

2) Priming of instrument (optional): After pumping reagents for at least fifteen minutes and prior to placing the first tray, an impression of the performance of the instrument can be initially obtained by running some standards or plasma extracts from the previous run.

2.3.3. Analytical Run

A) Adjustment of Instrument Response Using S_3 Standard

1) Set up the first tray according to the sequence indicated in Tables 5 and 6 and begin sampling. Adjust the cholesterol recorder pen to trace zero on the chart paper when the second and third zeolite treated isopropanol peaks appear. When the first S_3 cholesterol peak appears, monitor the recorder trace very carefully and as soon as the pen starts downward, push

the green "Start Print" button on the cholesterol channel of the digital printer, then push the white "Advance Print" button the the same channel for about five seconds (five blinks of the button light) and release. This sets the location of the reading point of the digital printer on the plateau of the recorder peak and also synchronizes the digital printer with the recorder. When the second S_3 cholesterol peak appears, make any correction necessary to bring the recorder peak to 60% of full scale deflection and digital display values to 300 mg/100 ml. This is accomplished by using the "Standard" calibration potentiometer on the colorimeter and the "Cal." control on the cholesterol channel of the digital printer. Adjust the triglyceride recorder pen to trace zero on the chart paper when the second and third zeolite treated isopropanol peaks appear. When the first S_3 triglyceride peak appears, monitor the recorder trace carefully. As soon as the red pen begins to deflect downward, push the green "Start Print" button on the triglyceride channel of the digital printer, then push the white "Advance Print" button for five seconds. This sets the location of the reading point of the digital printer on the plateau of the recorder peak and also synchronizes the digital printer with the recorder. On the second S_3 triglyceride peak, make any corrections necessary to bring the recorder peak to 50% of full scale deflection and the digital display to 200

mg/100 ml. This is accomplished by using the "Standard" calibration potentiometer on the fluoronephelometer, and the "Cal" control on the triglyceride channel of the digital printer.

2) The sequence from here on may not be interrupted by a "sample hold" maneuver because this results in loss of synchronization between the digital printer and the recorder. Linearity of the cholesterol and triglyceride standard curves is established by insuring that the S_1, S_2, S_3 and S_4 readings fall within the ranges in the following table:

Cholesterol	Recorder Pen	Visual Display	Acceptable Range *
Standard #1	20%	100	97 - 101
Standard #2	40%	200	196 - 202
Standard #3	60%	300	295 - 303
Standard #4	80%	400	394 - 404

Triglyceride	Recorder Pen	Visual Display	Acceptable Range *
Standard #1	12.5%	50	46 - 50
Standard #2	25%	100	96 - 100
Standard #3	50%	200	194 - 200
Standard #4	75%	300	293 - 301

If the criteria for linearily are met, proceed to the serum calibration below.

B) Use of Serum Calibrator for Cholesterol Analyses

1) Plasma cholesterol values determined on the AAII are 10-15% higher than those determined by a reference method (Abell, L.L. et al, J. Biol. Chem. 195:357, (1952)). Plasma samples usually contain a fairly

* These "acceptable range" values were determined from observations of performance characteristics of the systems in the LRC program.

constant proportion of free and esterified cholesterol. However, the total color yield of cholesterol ester is somewhat higher than that of free cholesterol. In the present procedure, plasma samples are being referred to a standard which contains only free cholesterol. It has proved possible to correct cholesterol values obtained on the AAII to reference Abell-Kendall values by use of a serum calibration sample (supplied to the LRC project by CDC, Atlanta, Ga.) which relates the observed value to the reference value.

2) Two extracts of the serum calibration pool (SERCAL) are prepared for each run. Place these extracts of the serum calibrator in positions #12 and 13 of the first tray (see Table 5).

3) Place S_3 standard in positions #14 and 15.

4) If the mean and the range of the observed SERCAL values are within control limits (sec. 3.1.2.), calculate the Printer Set Value (PSV) as follows:

$$PSV = \frac{\text{Abell-Kendall Value}}{\text{Mean AAII Value}} \times 300$$

A table of observed AAII SERCAL values, listing the corresponding PSV, facilitates this calculation.

5) When the peak corresponding to the S_3 standard in position 15 appears on the chart and printer, change the printer to display the PSV using the "Calibration" control on the cholesterol channel of the digital printer.

6) Quality control and other samples are analyzed con-

currently in positions 16-39. The recorder traces "uncorrected values" and the digital printer records "corrected values".

C) Readjustment of Recorder Response to S_3 Standard

When more than one tray of samples is run, the zeolite treated isopropanol in positions 2 and 3 are used to reset the baselines and the S_3 standard in position 5 (see Table 5) is used to readjust the recorder response to cholesterol and triglyceride if necessary. Correct the recorder scale settings by using the "Zero" and "Cal" controls on the colorimeter and fluoronephelometer. The digital printer reading point location is corrected using the "Advance Print" or "Retard Print" controls on the printer.

Note: Do not reset the digital printer. The printer responds to the recorder, thus, adjustment of the recorder response to S_3 automatically corrects the digital display.

2.3.4. Triglyceride Blanks

A) Modify the triglyceride cartridge according to Figure 3 using the two three-way valves and glass or Kel F tubing of approximately 0.060" internal diameter. This allows the hydrolysis heating bath to be readily bypassed.

B) Determine Triglyceride Blanks as Follows:

1) Divert the stream to bypass the hydrolysis heating bath and pump reagents for ten minutes.

2) Samples are arranged on the tray in the following

order: "Marker", three zeolite treated isopropanol blanks, and samples on which blank values are to be determined.

3) A convenient marker is a solution of glycerol (10 mg/100 ml) in isopropanol. It is used to resynchronize the digital printer with the recorder.

4) The isopropanol baseline is adjusted using the second and third isopropanol blanks.

5) Sample blank values are obtained from the digital printer and are subtracted from the total triglyceride values to obtain the net triglyceride result.

2.3.5. Treatment of Samples Giving off Scale Instrument Response (see sec. 2.2.5.)

2.3.6. Shutdown Procedure

A) After the last sample has been recorded, turn off the sampler, fluoronephelometer and colorimeter and Haake bath.

B) Turn off "Print" and channel A and B buttons on the digital printer.

C) Turn off digital printer and recorder.

D) Pump all manifold lines dry (fifteen minutes).

E) Pump 0.15% Brij-35 in distilled water through the triglyceride tubes for fifteen minutes, followed by distilled water through all tubes for fifteen minutes.

F) Once each week, pump 0.25 N hydrochloric acid containing 0.15% Brij-35 through the triglyceride tubes for fifteen minutes followed by distilled water for fifteen minutes.

G) Pump all manifold lines dry.

H) Remove platen.

2.3.7. Computation of Results

Results are read directly in mg/100 ml from the digital printer tape. In the event the digital printer is not available, results may be taken individually from the recorder tracing and converted to mg/100 ml. In this case cholesterol results are corrected manually by multiplication of the observed value by the factor

$$\frac{\text{Abell-Kendall Value of SERCAL}}{\text{Observed AAII Value of SERCAL}}$$

3. Quality Control of Cholesterol and Triglyceride Determinations.

An identical system of internal and external quality control and surveillance is used in each LRC laboratory in order to stabilize performance over the entire project and to assure that each laboratory will obtain results comparable to those found in the other laboratories.

Pure primary standards in solution as well as control pools of known triglyceride and cholesterol content, are available to each laboratory. It is hoped that the stable, comparable and precise values which are the goal of these laboratory procedures will also be accurate values approaching the true cholesterol and triglyceride content of the samples.

A quality control system is a method of operation which permits the current assessment of the quality of the work performed by a laboratory. The quality control system used by the LRC program includes analysis of 1) primary standards, 2) known serum control pools, and 3) unknown external quality control samples.

The procedures described are applicable to both AutoAnalyzer™ I and AutoAnalyzer™ II operations.

3.1. Internal Quality Control

Internal quality control involves the use of combined standard solutions containing cholesterol and triolein, and of quality control sera, the lipid concentrations of which are known to the operator and the laboratory manager.

3.1.1. Primary Standards

Clinics receive five solutions of cholesterol and triolein in isopropanol from the Lipid Standardization Laboratory, CDC, Atlanta, Ga. The concentrations of these five standards are:

	mg/100 ml	
	Cholesterol	Triolein
S1	100	50
S2	200	100
S3	300	200
S4	400	300
S5	50	0

The standards are all treated as far as possible in the same way as the samples. The final water concentration of both is 5%, and both samples and standards are treated similarly with zeolite mixture. The standards are used to compute the concentrations of cholesterol and triglycerides.

Each LRC laboratory keeps records of the machine response of all standards. These reveal any changes in chemistry or machine function from run to run.

Criteria for deciding whether to proceed with the analysis include the linearity of the standard curves and the stability of the reagent baseline. These data and the analysis of the control samples will enable the laboratory to maintain high quality performance.

3.1.2. Quality Control Samples and Construction of Quality Control Charts

A) Quality Control Samples

The most important component of the internal quality control system is the regular use, by all the LRC laboratories, of frozen quality control serum samples prepared from common pools for which total cholesterol and triglyceride concentrations are known. These are supplied to the LRC laboratories by the Lipid Standardization Laboratory, CDC, Atlanta, Ga. and are used over an extended

period of time. Since the total cholesterol and triglyceride concentrations are known*, accuracy (or proximity to the "true value") can be continuously monitored at all clinics. These samples also allow the estimation of within run and overall precision in the individual laboratories, and for all the LRC laboratories as a group.

Two of these pools are used to control the analysis of total plasma cholesterol and triglycerides and of ultracentrifugal bottom fractions ($d > 1.006$), and contain normal and elevated concentrations of cholesterol and triglycerides.

The preparation of zeolite-treated extracts of the control plasma pools is described in section 2.1.5. and is identical with the preparation of unknown plasma samples. REGARDLESS OF THE NUMBER OF SAMPLES ANALYZED IN A WORKING DAY, FOUR NORMAL AND FOUR HIGH QUALITY CONTROL SAMPLES ARE DISTRIBUTED THROUGHOUT THE RUN. (See Table 3 for AAI and Table 6 for AAII). Calculations of quality control limits for each pool are simplified by keeping the number of analyses of each pool constant.

Analysis of samples which contain lower cholesterol concentrations than those encountered in unfractionated human plasma (i.e. ultracentrifugal top fractions ($d < 1.006$,) and HDL cholesterol samples, see sec. 4) are controlled by using quality control samples from two pools,

* Determined by the LSL, CDC Atlanta. Total cholesterol by a modification of the method of Abell, et al. (Abell, L.L., et al, J. Biol. Chem. 195:357, (1952)). Triglyceride by a modification of the method of Carlson (Carlson, L.A., J. Atheroscler. Res. 3:334, (1963)).

one with a total cholesterol and the other with an HDL cholesterol concentration of about 50 mg/100 ml.

Two extracts of the first of these pools are used to monitor the analysis of cholesterol at this low concentration. Two extracts of the other pool are used to monitor the precision of the HDL separation procedure (see section 4.3.).

Ultracentrifugal top fractions ($d < 1.006$) and HDL cholesterol samples are analyzed in a separate section of the normal run. These analyses are considered separately for quality control purposes. The portion of the run containing these samples is preceded by a 50 mg/100 ml cholesterol standard (S_5) which is used to check instrument calibration (AAII) or instrument response (AAI) at this concentration. The four quality control extracts are interspersed randomly among the samples and are used to control these analyses in the same manner as the "normal" and "elevated" pools are used to control the analyses of total cholesterol, triglyceride, and ultracentrifugal bottom fractions ($d > 1.006$).

B) Construction of Control Charts

The construction of quality control charts is explained in the following reference: E.L. Grant, Statistical Quality Control, Second Edition, (1952), New York, McGraw-Hill Book Company.

Control charts should be maintained for all quality control pools, and for the serum pool used to calibrate the AAII (see section 2.3.3.B.).

Control charts are maintained from the first day of operation by making separate plots for each constituent of each pool. It is convenient to plot the statistics $\bar{X}$ (the average of the analyses on a single pool) and R (the range or difference between the highest and lowest value for that pool) on the same chart. The statistic ($\bar{X}$ or R) is plotted on the vertical axis and the date the determination was made on the horizontal axis (figure 4).

The scale should be greatly expanded to provide a clear picture of variation and trends.

After collecting the data for twenty runs, preliminary control limits can be computed as indicated in the next section. More permanent control limits are calculated after fifty runs and checked after each additional twenty runs.

C) Calculation of Control Limits

1) $\bar{X}$ Chart

These charts are maintained for all quality control pools and for the serum calibration pool. The control charts for cholesterol analysis of quality control pools performed on the AAII reflect corrected values (see section 2.3.3.B.) whereas the control limits on the calibration pool are based on the observed values.

Step 1: Compute the Standard Deviation ($S_{\bar{x}}$) of a Daily Mean ($\bar{X}$)

a. $$S_{\bar{x}}^2 = \frac{\text{Sum of Squares of Daily Means} - \frac{\text{Sum of Daily Means Squared}}{\text{Number of Days}}}{\text{Number of Days} - 1}$$

$$+ \frac{\Sigma \bar{X}^2 - \frac{(\Sigma \bar{X})^2}{n}}{n - 1}$$, where n = number of days data have been collected.

b. $$S_{\bar{x}} = \sqrt{S_{\bar{x}}^2}$$

Step 2: Compute the Overall Mean ($\bar{\bar{X}}$)

$$\bar{\bar{X}} = \frac{\text{Sum of Daily Means}}{\text{Number of Days}}$$

Step 3: Compute Control Limits

Center line = $\bar{\bar{X}}$

control limits (2 sigma) = $\bar{\bar{X}} \pm 2\, S_{\bar{x}}$

control limits (3 sigma) = $\bar{\bar{X}} \pm 3\, S_{\bar{x}}$

These limits are indicated on the $\bar{X}$ chart with horizontal lines intersecting the vertical axis at the computed values (see Figure 4).

2) R Chart

Step 1: Compute the Average Range ($\bar{R}$)

$$\bar{R} = \frac{\text{Sum of Range Values}}{\text{Number of Days}} = \frac{\Sigma R}{n}$$

Four analyses are performed for each run on each pool used to control the analysis of total cholesterol and triglyceride and ultracentrifugal bottom fraction ($d > 1.006$). The

control limits for the R chart are computed as follows:

Step 2: Compute Control Limits

Center line = $\bar{R}$

UCL (2-Sigma) = (1.855) $\bar{R}$

UCL (3-Sigma) = (2.280) $\bar{R}$

These limits are indicated on the R chart with horizontal lines intersecting the vertical axis at the computed values (see Fig. 4).

Two analyses are performed for each run on each of the pools used to control the analysis of HDL cholesterol and ultracentrifugal top fractions (d < 1.006) as well as the serum calibration pool. In this case, control limits are computed as follows:

control limit (2-Sigma) = (2.46) $\bar{R}$

control limit (3-Sigma) = (3.27) $\bar{R}$

D) Additional Information to be Indicated on Control Charts

Standardization of lipid determinations according to CDC criteria requires that the mean of multiple analyses ($\bar{X}$) of quality control pools falls within certain limits. These limits are established by the Lipid Standardization Laboratory:

1) Cholesterol

control limit = TV $\pm$ (.05)TV

2) Triglycerides

Target Value (mg/100 ml)	Control Limits (mg/100 ml)
0 - 249	TV ± 9
250 - 349	TV ± 13
350 - 399	TV ± 15
400 - 499	TV ± 17

These limits should be indicated on the $\bar{X}$ chart.

3.1.3. Use of Quality Control Charts

A) Stability of Pattern

No system of surveillance is completely effective until a stable pattern is achieved. The initial set of data (first twenty runs) should be checked for evidence of an unstable pattern. If, after plotting $\bar{\bar{X}}$ on the $\bar{X}$ chart, either 1) seven run means in succession, or 2) eight of nine means from successive runs fall either all above or all below $\bar{\bar{X}}$, the system <u>was not stable</u> during the period of data collection. Efforts are then made to stabilize the system. It should be noted that control limits constructed with data collected when a stable pattern does not exist will be of limited usefulness. Once a stable pattern has been achieved control limits should again be computed using data from the stable period.

In the experience of the LRC laboratories there appears to be a "cycling effect" with the AutoAnalyzer™ systems in which assayed values on control pools may tend to fluctuate above or below the mean for periods in excess of the criteria stated above. The reasons for this cycling are unclear but may be related to differences in reagents or pump tubing which show up over a long period of time. For this reason the assumption of complete sta-

bility of the systems which is inherent in the adoption of the criteria above may not hold and the use of these criteria may only serve as a warning signal to the laboratory director that there may be some instability in the system.

B) Acceptability of Results

Once control limits have been established the system should be declared "out of control" if one or more of the following events occur:

1) $\bar{\bar{X}}$ Chart

a) A single daily mean ($\bar{\bar{X}}$) falls outside the 3-sigma limits.

b) Two successive $\bar{\bar{X}}$ values fall between the 2-sigma and 3-sigma limits.

c) In a system for which the criteria of stability (3.1.3.A. above) are satisfied, seven $\bar{\bar{X}}$ values in succession fall either all above or all below the center line ($\bar{\bar{X}}$).

2) R Chart

a) A single R value falls outside the 3-sigma limits.

b) Two successive R values fall between the 2-sigma and 3-sigma limits.

c) Seven R values in succession fall above the center line $\bar{R}$.

C) Criteria for Standardization Limit

The accuracy requirements of the LSL are not being met if either five run means in succession or six of seven run means in succession fall outside the standardization limits even though the run means may have been

within the internal quality control limits of the laboratory (sec. 3.1.2.D.). Under these conditions LRC project samples are not analyzed.

3.1.4. Duplicate Determinations on Subject Samples

The precision of the entire process can be monitored from the time the sample is drawn by drawing duplicate blood samples on 2% of the patients and performing complete duplicate analyses. The two samples should be analyzed on the same day. If, after three months trial, the difference between duplicate drawings is acceptable, this check can be discontinued.

As an additional check, approximately 3% of the patient samples may be reanalyzed on a different day. The range of values obtained from the replicate analyses may then be compared with the variation exhibited by the quality control samples. This replication may or may not be blind to the analyst. If after three months the variation observed with fresh samples is similar to that found with quality control samples, this procedure may be discontinued.

3.2. External Quality Control (see also sec. 3.3.)

See LRC protocol section VIII, "Design and purpose of cholesterol and triglyceride standardization programs for Lipid Research Clinics".

For the LRC project, external quality control (surveillance phase standardization) consists of the analysis of six samples per week by each laboratory. These samples are of unknown cholesterol and triglyceride concentration and are furnished by the CDC Lipid Standardization Laboratory. Results will be reported as described in section 3.3.3.

3.3. Standardization of Lipid Determinations

3.3.1. Initial Standardization and Surveillance

A) Each of the LRC laboratories participates in the CDC lipid standardization program prior to becoming operational. The standardization is divided into an evaluation phase (part I), a standardization phase (part II) and a surveillance phase (part III).

B) Purpose of Each Phase

Part I, the evaluation phase, covers a short period of time during which the precision and accuracy of cholesterol and triglyceride analyses are established for each laboratory. Its purpose is to identify problems related to the analyses and to determine if performance is satisfactory for entrance into part II. Part I consists of the analysis of three pools of unknown and two pools of known cholesterol and triglyceride concentration levels. Six analyses are performed on samples from each pool in each of four runs over a period of two to four weeks. A single analysis is performed on each sample. The standardization program offers frozen reference serum samples with known cholesterol and triglyceride concentrations to laboratories which do not successfully complete part I. When the laboratory performance has improved it may reenter part I.

Part II, the standardization phase, is a more intensive evaluation of the laboratory's performance. Its purpose is to determine precision and accuracy more thoroughly and to document that analyses remain stable over a longer

period of time. Three sets of frozen serum samples (a total of 320) are analyzed on thirty-two different days, within a period of eight to sixteen weeks. During this period of time, results must continue to fall within acceptable limits. Upon satisfactory completion of part II, the laboratory is designated as "standardized".

Part III, the surveillance phase, provides a continuous check of performance after standardization. Twenty-four samples of unknown lipid concentration are analyzed in four runs each four to five week period. Laboratories performing satisfactorily continue to be designated as "standardized".

C) Criteria for Acceptable Performance

Criteria for acceptable performance of cholesterol and triglyceride analyses are given in Table 7.

3.3.2. Standardization of Laboratories which Change Location

If an LRC laboratory moves after completing the standardization phase, the CDC may require the laboratory to participate in a short term evaluation of the accuracy and precision of cholesterol and triglyceride analyses. Changes in long-term precision will be detected during regular part III surveillance.

3.3.3. Reporting Quality Control Data

General

Periodically the director of each LRC laboratory will send quality control data to the Central Patient Registry and Coordinating Center at Chapel Hill, North Carolina, and

to the Lipid Standardization Laboratory. Data will be sent on forms provided by CPR and will include all analytical runs performed in the laboratory, whether or not the runs are accepted.

Frequency of Reporting

A report is made to the CPR every two weeks. Results are recorded on CPR Bi-weekly Quality Control Report forms (see Appendix II). Part III standardization results are reported to CDC once each month (see Appendix II).

4. Quantitation of the Lipoproteins.

4.1. Appearance of Plasma

4.1.1. Appearance of Freshly-Mixed Plasma

After the plasma is placed in the 20 ml storage vial and mixed, the clarity/turbidity/frank lactescence, and the normal or icteric nature of the plasma may be recorded if required.

4.1.2. Standing Plasma Test

A 2 ml aliquot of plasma is removed from the 20 ml storage vial, placed in a 10 x 75 mm glass test tube and stored undisturbed for at least sixteen hours at 4°C. The plasma is examined for the presence of a supernatant chylomicron layer, using uniform lighting conditions. Chylomicrons are judged to be either present or absent. At the same time the infranate is examined and is judged to be either clear or turbid. Results are recorded on the appropriate form.

4.2. Ultracentrifugal Separation of Lipoproteins

4.2.1. Source of Sample

The sample used is freshly drawn plasma which must not be frozen. If it is necessary to transport specimens to the laboratory they should be shipped, unfrozen, packed in wet ice (sec. 1.5.). Ultracentrifugation should be carried out as soon as possible, but samples may be stored at 4°C for up to five days if necessary.

4.2.2. Equipment and Supplies

A) Ultracentrifuge

The following description is based upon use of the Beckman Preparative Ultracentrifuge, Model L2-65 (or

later equivalent).

B) Rotor and Tubes

The model 40.3 rotor is used with cellulose nitrate tubes, 2.5" x 0.5" and aluminum caps. Polyallomer tubes may crack when sliced and cause error in subsequent analyses. If these tubes must be used, the analyst should be aware of this problem.

C) Tube Slicer

The tube slicer is constructed of stainless steel and acrylic polymer and is furnished with sets of rubber gaskets which accommodate tubes 1/2", 5/8" and 1" in diameter.

D) 0.15 M Saline*

0.15 M saline is prepared by dissolving 8.77 g of dry reagent grade sodium chloride in distilled water and diluting to one liter in a volumetric flask at 23°C. Cool to 4°C for use.

E) 2.5 ml and 5 ml syringes with 20 gauge 1-1/2" needles. The point of the needle can be removed to facilitate aspiration of the samples.

4.2.3. Ultracentrifugation

A) Pre-cool the 40.3 rotor to 10°C in the ultracentrifuge.

B) Label cellulose nitrate tubes with a waterproof marker and place them in a test tube rack in an ice bath.

C) Assemble the centrifuge tube caps except for the fill-hole set screw.

* 0.15 M saline is used by the LRC laboratories as a matter of convenience and appears to give satisfactory results. It should be noted that 0.15 M saline (8.77 g NaCl/l) has an actual density of 1.0044(20°C) whereas a solution which has a true density 1.0063(20°) is obtained using 0.195 M saline (11.42 g NaCl/l)

D) Allow samples to warm to room temperature (approximately 23°C) and pipet 5.0 ml of plasma into the centrifuge tubes (2.5 ml of plasma may be used if necessary). These transfers are made using only class A pipets.

E) Carefully layer 0.15 M saline on the sample to within 5 mm of the top of the centrifuge tube.

F) Cap the tubes tightly. Fill the tubes completely with saline using a syringe with a 26 gauge needle and seal the tube by inserting the fill-hole set screw. Gently squeeze the sealed tube to check for leaks.

G) Place the tubes in the rotor sockets in such a way that the rotor is precisely balanced.

H) Place a very thin film of vacuum grease on the rotor gasket and seal the rotor.

I) Centrifuge samples for eighteen hours at 10°C at 105,000 x g (40,000 r.p.m.). (Centrifugation at 10°C prevents the samples from freezing, as occasionally occurs if the centrifuge is set at 4°C. Use of the higher temperature causes no apparent changes in the lipoproteins).

4.2.4. Preparation of Ultracentrifugal Fractions

A) Label and chill 5 ml volumetric flasks.

B) Stop centrifuge after eighteen hours. Do not use brake.

C) Gently remove rotor. The area where the tubes are sliced should be as close as possible to the centrifuge.

D) Using the extraction tool, remove each tube slowly. Avoid any abrupt movement.

E) Insert the tube into the tube slicer until the bottom edge of the cap is 0.8 cm from the top of the tube slicer and

the small dent in the tube faces away from the point of the slicer blade. For very lipemic samples this distance may have to be increased so that the tube is sliced in the clear zone below the supernatant lipid layer.

F) Remove the set screw from the fill-hole. Cover the hole with a finger. Slice the tube with a quick, smooth thrust of the blade.

G) One of the following procedures is used to prepare ultracentrifugal top fractions ($d < 1.006$).

1) Cholesterol analysis and electrophoresis. Withdraw as much as possible of the top fraction through the fill-hole using a 2.5 ml disposable syringe or pipet. Transfer to a 5 ml glass stoppered volumetric flask. Replace the set screw and remove the top portion of the tube, with the cap, from the tube slicer. Transfer the remainder of the top fraction to the volumetric flask. Rinse the slicer and cap with 0.15 M saline, add the washings to the volumetric flask, and bring the contents to volume with 0.15 M saline. Mix by inversion, transfer the samples to 20 ml storage vials and store the vials at $4^{\circ}C$.

2) Electrophoresis only. Withdraw as much as possible of the top fraction through the fill-hole using a 2.5 ml disposable syringe or pipet. Transfer the fraction to a storage vial. Replace the set screw, remove the top portion of the tube, with the cap, from the slicer and rinse it with a small amount of sample from the

storage vial. Return the sample to the storage vial. Repeating this process, rinse the material remaining in the well of the tube slicer. Again return the material to the vial. The vial is capped and is stored at 4°C.

H) Withdraw the blade slightly and remove about 1 ml of the bottom fraction, using a 5 ml disposable syringe or pipet and transfer it to a 5 ml volumetric flask.

I) Withdraw the blade completely and work the bottom portion of the tube from the slicer.

J) Loosen all material from the wall of the tube with the syringe needle or pipet tip and transfer the remainder of the bottom fraction to the 5 ml volumetric flask.

K) Rinse the bottom portion of the tube with a small amount of 0.15 M saline and add the washings to the 5 ml volumetric flask. Bring the contents of the flask to volume with 0.15 M saline.

L) The flasks are placed in an ice bath for thirty minutes and their contents are periodically mixed by gentle inversion. The samples are then transferred to 20 ml storage vials and stored at 4°C.

4.2.5. Extraction and Analysis of Ultracentrifugal Fractions

A) The ultracentrifugal fractions are analyzed for cholesterol in the same manner as unfractionated plasma (sec. 2.1.5.). Inasmuch as the cholesterol content of ultracentrifugal top fractions ($d < 1.006$) is normally quite low, the sensitivity of AAI analyses ($FeCl_3$ reagent) is increased two-fold by

utilization of a 1:10 isopropanol extract rather than the usual 1:20 extract. One-half ml of sample is brought to 5.0 ml with 99% isopropanol and treated with zeolite mixture. Increase in the sensitivity of the test in this way is possible because the color yield of the chromophore produced from cholesterol is not markedly affected by changes in water concentrations of the extract between 90% (1:10 extract) and 95% (1:20 extract). The color yield with the AAII system (Lieberman - Burchard reagent) is, however, quite sensitive to changes in the water content of the extract. Therefore 1:20 extracts are used for analysis of ultracentrifugal top fractions ($d < 1.006$) with this instrument.

B) On both systems, ultracentrifugal bottom fractions ($d > 1.006$) are analyzed as 1:20 extracts.

C) As with unfractionated plasma (section 2.2.5.) a fraction giving an off-scale cholesterol response is diluted with 0.15 M saline and the analysis is repeated.

D) The analysis of ultracentrifugal fractions is performed and controlled as described in sections 2 and 3.

4.3. Separation of HDL Cholesterol

4.3.1. Reagents

A) Manganese Chloride Solution, 1 M

Add 197.91 g of $MnCl_2,4H_2O$ to a one liter volumetric flask and dilute to volume with distilled water.

B) Heparin, 5,000 units/ml

1.0 ml heparin (40,000 units/ml) is diluted to 8 ml with 0.15 M saline. Note: Heparin preparations should be checked for their ability to completely precipitate

LDL and VLDL since incomplete precipitation has been observed with some heparin preparations. Completeness of precipitation can be readily assessed by electrophoresis (section 5) of the heparin-Mn^{2+} supernatant.

4.3.2. Procedure

A) Transfer 3.0 ml of plasma to a 12 ml conical glass centrifuge tube using a volumetric pipet.

B) Add 120 ul of heparin solution (5,000 units/ml) using a 250 ul syringe. Mix using a vortex mixer.

C) Add 150 ul of 1 M $MnCl_2$ using a 250 ul syringe, and mix immediately using a vortex mixer.

D) Let the samples stand for exactly thirty minutes in an ice bath.

E) Sediment the precipitate at 1500 x g for thirty minutes at 4°C.

F) Carefully transfer the clear supernatant HDL solution for analysis to a 20 ml storage vial. If the sedimentation of the precipitate is incomplete, as occurs when samples have markedly elevated concentrations of triglycerides, the heparin-manganese precipitation is performed on the ultracentrifugal bottom fraction ($d > 1.006$) (sec. 4.2.).

G) Variation:

One or 2.0 ml of plasma can be used, if necessary, by proportionately reducing the volumes of heparin and $MnCl_2$ solutions added. Alternatively the 1.0 or 2.0 ml samples can be diluted to 3.0 ml with 0.15 M saline and treated as described above.

4.3.3. Preparation for Cholesterol Determination on AAI and AAII

A) AAI

Extracts (1:10) of HDL samples are prepared and treated as described for ultracentrifugal top fractions ($d < 1.006$) analyzed on the AAI (section 4.2.5.A.).

B) AAII

Extracts (1:20) of HDL samples are prepared and treated as described for ultracentrifugal top fractions ($d < 1.006$) analyzed on the AAII (section 4.2.5.A.).

C) Quality control of HDL cholesterol analysis is described in section 3.1.2.A.

4.4. Calculation of Cholesterol Content of HDL, LDL and VLDL

4.4.1. HDL Cholesterol

A) AAI

The concentration of HDL cholesterol determined on 1:10 extracts must first be divided by 2, then multiplied by the factor 1.09 to account for the dilution which occurs upon addition of the heparin and Mn^{++} solutions. The result is the concentration of HDL cholesterol in mg/100 ml plasma.

B) AAII

HDL cholesterol is determined on 1:20 extracts. The observed concentration is multiplied by the factor 1.09 to correct for dilution which occurs on addition of heparin and $MnCl_2$ solutions. The result is the concentration of HDL cholesterol in mg/100 ml plasma.

C) When the plasma has been diluted before precipitation, the appropriate factor should be used to calculate HDL cholesterol concentration in plasma.

4.4.2. LDL Cholesterol

The ultracentrifugal bottom fraction ($d > 1.006$) contains both HDL and LDL. Therefore the LDL cholesterol concentration is obtained by subtracting HDL cholesterol from the cholesterol contained in the entire ultracentrifugal bottom fraction ($d > 1.006$).

LDL cholesterol = ultracentrifugal bottom fraction cholesterol - HDL cholesterol (all expressed in mg/100 ml).

4.4.3. VLDL Cholesterol

VLDL cholesterol is calculated as the difference between the total plasma cholesterol concentration and that of the ultracentrifugal bottom fraction ($d > 1.006$).

VLDL cholesterol = total cholesterol - ultracentrifugal bottom fraction cholesterol (all expressed in mg/100 ml).

VLDL cholesterol is contained in the ultracentrifugal top fraction ($d < 1.006$) along with any chylomicron cholesterol. Since it is sometimes difficult to completely recover the ultracentrifugal top fraction, it is not used as a measure of VLDL but only as a check on the ultracentrifugal procedure.

5. Lipoprotein Electrophoresis.

5.1. General

For purposes of the LRC Project, lipoprotein electrophoresis is performed to establish the presence of "floating beta" lipoprotein for the assignment of the Type III pattern (see sec. 9.) and to test for the presence of lipoprotein lipase using the Intravenous Heparin Strip Test.

"Floating beta" lipoprotein is present if a lipoprotein species of beta mobility (see Figure 5) is observed in the ultracentrifugal top fraction ($d < 1.006$). In order to compare the electrophoretic mobility of the lipoproteins in an individual sample, electrophoretic patterns are also obtained on unfractionated plasma and on ultracentrifugal bottom fraction ($d > 1.006$).

Either paper or agarose gel electrophoresis may be used. Cellulose acetate, starch block and polyacrylamide gel electrophoresis are not considered suitable for LRC purposes.

The following methods have been found satisfactory for plasma lipoprotein electrophoresis and are included for information.

5.2. Paper Electrophoresis*

A) General

Electrophoresis is performed in albumin containing barbital buffer pH 8.6. on Whatman #1 filter paper strips. After electrophoresis the strips are dried and stained with Oil Red O dissolved in 60% ethanol. Oil Red O stains cholesterol esters and triglyceride present in the separated lipoproteins.

B) Reagents

1) 0.12 M barbital buffer, pH 8.6. containing 0.001

* Description kindly furnished by Betty Masket, M.S. Lipid Metabolism Branch, NHLI, Bethesda, Maryland

M disodium ethylenediamine tetraacetate.

20.6 g sodium barbital (MW = 206.18)
3.68 g barbital, acid (MW = 184.20)
and 0.372 g disodium ethylenediamine tetraacetate (MW = 372.254)

is made to one liter with distilled water. The pH of the solution is 8.6 ± 0.1. Just before using add 10 g/l bovine serum albumin (Fraction V).

2) Oil Red O stain. 1 g Oil Red O is dissolved in 1.5 liters of ethanol in a flask equipped with a reflux condenser and heated using a heating mantle. One liter of distilled water is added through the condenser in five or six portions, allowing the mixture to reflux after each addition. When all the water has been added, the mixture is refluxed for at least one hour. The mixture is cooled, stored, and used at 37-40°C. The temperature should not fall below 37°C. If the mixture cools before it is used, it may be refluxed for one hour and cooled to 37°C.

C) Preparation of Electrophoresis Cells

Paper electrophoresis is carried out at room temperature on Whatman #1 paper strips using the Durrum type electrophoresis cell with a constant voltage power supply.

The paper strips are prepared for electrophoresis by marking the origin with a medium lead pencil, moistening with buffer from the cell, and equilibrating for three or four hours (never less than one hour) before the samples are applied.

D) Procedure

After equilibration the strips are loaded. Twenty ul of unfractionated plasma, 20 ul of ultracentrifugal bottom fraction

(d$>$1.006), and 40 ul of ultracentrifugal top fraction (20 ul if lipemic) are used. Both ultracentrifugal fractions should be run concurrently to facilitate the identification of "floating beta" lipoprotein. Electrophoresis is performed at room temperature for sixteen hours at constant voltage (120 V). This results in a current flow of 6 to 8 mA per cell at the beginning of the analysis and 7 to 11 mA per cell at the end. The strips are subsequently dried at 90°C for twenty to thirty minutes, stained with Oil Red O for approximately one to one and a half hours, rinsed with tap water for one minute, blotted, air dried, and examined visually.

E) Discussion

Albumin is added to the cell buffer to improve the separation and definition of the lipoprotein bands. If it is omitted the lipoproteins are not sharply defined but appear as a smear from the origin to a point beyond the beta lipoprotein band.

The buffer may be used for two months or longer provided the polarity of the cell is changed each run. The cells are kept at room temperature at all times. When freshly prepared buffer is used, the lipoprotein bands often are not sharp. This difficulty may be overcome by using old buffer to moisten the strips before placing them in the cell containing the freshly prepared buffer.

The sample may be kept at 4°C for several days before electrophoresis. It should never be frozen, since freezing irreversibly alters some lipoprotein patterns. After a single freezing and thawing, plasma rich in prebeta lipoprotein displays a large artifactual "chylomicron" band at the origin. If the plasma, especially when lipemic, is allowed to stand at room temperature

for several hours, denatured material accumulates at the origin and the mobility of the lipoprotein bands is increased.

The electrophoretic patterns of lipoproteins are subject to alteration from a number of variables. One must observe the following precautions in order to minimize this variation:

1) Good contact must be made between the strips and the wicks.
2) The end wicks on the cell cover should be quite moist. Also, filter paper strips are placed on the inside of the cell cover as indicated in Figure 6. This minimizes the distortion which usually occurs on the end strip.
3) The fluid level in the cell must be the same on both sides and within the maximum and minimum fluid level lines scribed on the sides of the cell.
4) The opening on the top of the cell cover is covered with short overlapping pieces of three-quarter inch waterproof tape so that only one paper strip at a time is exposed when loading.
5) Electrophoresis should be performed in a draft-free area at a relatively constant temperature.
6) Salt accumulates on the baffle adjacent to the positive electrode. This should be carefully removed from the baffle with a spatula and returned to the same electrode chamber. Salt is not permitted to accumulate on the center baffle which separates the positive from the negative electrode chamber. Such an accumulation produces a salt bridge which results in damage to the power supply unit.

7) After each use, the dye mixture is heated to incipient boiling and allowed to cool on standing to 37-40°. Fresh dye mixture is periodically added to maintain the level of the dye bath. The mixture is discarded after two months and a new mixture prepared. When fresh dye mixture is used, the strips are stained from forty-five to sixty minutes. After about one week, the staining time is increased to one and one-half hours. The electrophoretic pattern of a normal subject is prepared and stained with each run as a measure of the variation of the staining procedure.

8) Ultracentrifugal top ($d < 1.006$) and bottom ($d > 1.006$) fractions should be run side by side in order to facilitate the identification of "floating beta" lipoprotein in the top fraction if it is present. If samples are not lipemic 40 ul of the top fraction are applied to the paper. Twenty ul of the bottom fraction and 20 ul of whole plasma are used (see section 5.1.D.).

F) References

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5.3. Agarose Electrophoresis

Acceptable results have been obtained either by preparing agarose media immediately before use or by using a commercially available system of prepared plastic slides coated with agarose gel. Both systems are described.

5.3.1. Laboratory Prepared System*

A) Reagents:

1) Barbital buffer, ionic strength 0.045, pH 8.6. Dissolve 7.13 g sodium barbital in 500 ml distilled water and adjust to pH 8.6 with concentrated HCl. Dilute to one liter with distilled water, mix and readjust pH, if necessary. Store at 4°C.

2) Sodium hydroxide 0.1 M. Four g NaOH are dissolved in distilled water and diluted to one liter.

3) Dye, Fat Red 7B (Color index No. 26050, Synonym: Sudan Red 7B) Stock: Add 225 mg fat red 7B to 946 ml absolute methanol, mix and allow to stand overnight before use. Store at room temperature. Working solution is freshly prepared for each use. Add 40 ml of 0.1 M NaOH to 200 ml of stock solution, mix, and add eight drops Triton X-100. Care must be taken to avoid contamination of other laboratory glassware with dye solution. The solution should not be used to stain more than three plates.

4) Clearing solutions:

* Kindly furnished by Dr. Gerald Kessler, Div. of Biochemistry, The Jewish Hospital of St. Louis, Mo.

a) 75% v/v solution of ethanol in water. Mix 150 ml of absolute ethanol with 50 ml of distilled water.

b) 2% glycerol (v/v) in distilled water.

c) 1% glycerol in 55% ethanol. Mix 110 ml ethanol, 88 ml water, and 2 ml glycerol.

5) Tracking Dye. Add a small amount of bromphenol blue dye (using the tip of a small spatula) to 2 ml of a control sample which will be used to follow the progress of the electrophoretic run.

6) Buffered 0.5% agarose gel. Place 100 ml barbital buffer and 0.5 g agarose in a 250 ml erlenmeyer flask. Place the flask in a beaker of boiling water and dissolve the contents with gentle stirring. Several electrophoresis plates can be prepared at the same time (see procedure). Plates are to be stored in moisture chamber at 4°C and should not be used after five days.

B) Equipment (see Appendix I)

C) Procedure:

1) Cut wicks and film and prepare agarose solution.

2) Pipet 15 ml of hot agarose solution onto film. Allow agarose to cool two-three minutes. Immediately place nine nipples horizontally on the agarose plate one inch from the edge to form the sample wells. The plates can then be stored in the moisture chamber. The nipples are carefully removed with forceps immediately before the samples are applied. Distortion

of the sample wells must be avoided.

3) Each well is filled with 5 ul of sample (plasma or ultracentrifugal fractions). Avoid overflow and bubble formation. Eight samples and one control sample containing Bromophenol Blue are run on each plate.

4) Forty ml cold barbital buffer are added to each electrode chamber (see Figure 7). The agarose plate is positioned in the electrophoresis cell. Two wicks are moistened with barbital buffer, blotted to drain the excess and one is placed in the buffer in each electrode chamber (nearest center of cell) and across the top edges of the agarose plate. The wicks must make direct contact over the entire length of the agarose plate. Fresh buffer is used for each electrophoretic run.

5) Electrophoresis is performed at a constant current of 45 mA for forty minutes or until the leading edge of the tracking dye is about seven-eighths inch from the point of origin. (Do not exceed 150 V or 50 mA).

6) The agarose plate is removed from the cell and washed for five minutes with 1% glycerol in 55% ethanol. The solution is gently agitated over the surface of the agarose plate at one minute intervals. After the glycerol wash the porteins are fixed at 70-80°C for one and one-half to two hours. Do not place agarose plate directly on the oven. The plates are placed on several layers of paper toweling to avoid direct

contact with hot metal of the oven racks.

7) Plates are cooled, stained for approximately ten minutes in freshly prepared Fat Red 7B solution, and rinsed for thirty seconds with 75% ethanol.

8) Plates are then immediately rinsed in 2% glycerol for thirty seconds and dried at 70-80°C (fifteen-twenty minutes).

9) Ultracentrifugal fractions from a particular sample should be run in adjacent sample wells to facilitate the identification of floating beta lipoprotein in the ultracentrifugal top fraction ($d < 1.006$).

D) Notes:

1) Plate size can be adjusted for the particular electrophoretic cell used.

2) Sudan Black B stain can be used instead of Fat Red 7B if desired.

E) References:

1) Noble, R.P., J. Lipid Res. 9:693-700 (1968)

2) Papadopaulos, N.M. and J.A. Kintzios, Anal. Biochem. 30:421-426 (1969)

3) Cawley, L.P., Electrophoresis and Immunoelectrophoresis, Copyright 1966 by American Society of Clinical Pathologists

4) Straus, R. and M. Wurm , Amer. J. Clin. Pathol., 29: 581 (1958)

5.3.2. Commercially Prepared System

A) A commercially available system of prepared plastic slides coated with agarose and shipped in barbital buffer has been found suitable for lipoprotein electrophoresis. Each slide accommodates three samples and is stained with Sudan Black.

B) Procedure. Follow instructions supplied with the system. Eighteen samples can be run simultaneously.

6. Determination of Free and Esterified Cholesterol.

6.1. General

The definition of obstructive liver disease or biliary cirrhosis, which may cause secondary dyslipoproteinemias, requires the separate measurement of plasma free and esterified cholesterol and of total lipid phosphorus expressed as total phospholipid. The physician will request this test when these diseases are suspected. The lipoprotein pattern of obstructive jaundice will be assumed if the ratio of plasma unesterified: total cholesterol is $>$ 0.32; and the plasma ratio of total cholesterol: phospholipid is $<$ 0.65 (Peters, J.P. & Man, E.B., J. Clin. Invest. 22:707 (1943)).

6.2. Determination of Unesterified: Total Cholesterol

The procedure should consist of the following (or equivalent) steps:

A) Extraction of 1 or 2 ml of plasma e.g. by the Folch extraction and washing method, (Folch, J., et al., J. Biol. Chem. 226: 497, (1957));

B) Concentration of the extract and quantitative application to a silica gel coated thin-layer plate which is then developed in a suitable solvent system (e.g. petroleum ether/diethyl ether/acetic acid 70/30/0.5 v/v). Twenty-five ul of a mixture containing 1 mg/ml cholesterol and 1 mg/ml cholesteryl oleate in ethanol or isopropanol should also be applied to the plate to identify the locations of the cholesterol and cholesteryl ester bands since the plasma extract contains a number of other substances;

C) Visualization with a non-destructive reagent such as water or a solution of rhodamine 6G in ethanol;

D) Removal of the esterified and unesterified-cholesterol bands, and quantitative transfer to small glass elution columns;

E) Elution from the silica gel with diethyl ether and evaporation of the two eluates;

F) Solution of the separated components in a known volume of isopropanol (95%) and determination of cholesterol content as described in section 2. Zeolite treatment is probably unnecessary, since both fractions are similarly treated, and only the ratio is of interest.

G) The ratio of unesterified to total cholesterol is calculated as follows:

$$\text{Ratio} = \frac{\text{unesterified cholesterol}}{\text{unesterified + esterified cholesterol}}$$

7. Determination of Phospholipid Phosphorus.

The procedure should consist of the following (or equivalent) steps:

A) Extraction of 1 ml of plasma (e.g. by use of the Folch extraction procedure and washing method (Folch, J., et al., J. Biol. Chem. 226:497 (1957))).

B) Transfer of a suitable aliquot of extract to a digestion tube and evaporation to dryness.

C) Digestion of phospholipid to inorganic phosphate and determination of phosphate by the method of Bartlett (Bartlett, G.R., J. Biol. Chem., 234:466 (1959)).

D) Expression of the total lipid phosphorus as "total phospholipid". Total lipid phosphorus is converted to "total phospholipid" (as phosphatidyl choline) as follows: Total phospholipid (mg/100 ml) = Total lipid phosphorus (mg/100 ml) x 25. The total cholesterol concentration of the sample is determined as described in section II. The ratio of plasma cholesterol: plasma phospholipid is calculated as follows:

$$\text{Ratio} = \frac{\text{plasma total cholesterol concentration (mg/100 ml)}}{\text{plasma phospholipid concentration (mg/100 ml}}$$

E) A single Folch extract can be used for determining both the total phospholipid concentration and the ratio of unesterified to total cholesterol (sec. 6.).

8. Intravenous Heparin Strip Test.

This test is used to distinguish between Types I and V hyperlipoproteinemia (Sec. 9). It is performed when a fasting plasma sample contains chylomicrons and when the concentration of plasma triglycerides exceeds 400 mg/100 ml (Fredrickson, D.S. and Levy, R.I. in Metabolic Basis of Inherited Disease, 3rd ed. Stanburry, J.B., Wyngaarden, J.B., and Fredrickson, D.S., Eds. McGraw-Hill Co., 1972, pp 545-614).

A fasting blood sample is obtained. Heparin (10 units/kg body weight) is then administered intravenously. Ten minutes later a second (post-heparin) blood sample is obtained. Both samples are subjected to lipoprotein electrophoresis (Sec. 5). If lipoprotein lipase is not deficient the lipoprotein bands of the post-heparin sample exhibit increased mobility, marked smearing in the beta - prebeta region and sharpening of the alpha band. These changes are seen with Type V patient samples. The patterns of post-heparin Type I patient samples undergo no change and have the same appearance as the pre-heparin samples.

Subjects with normal concentrations of plasma triglycerides also exhibit no change in lipoprotein pattern following the intravenous injection of heparin.

9. Lipoprotein Phenotyping System

9.1 General

For purposes of the LRC program hyperlipidemia is translated to hyperlipoproteinemia in accordance with the guidelines recommended in the Report of the National Heart and Lung Institute Panel on Hyperlipidemia and Premature Atherosclerosis, June 12-13, 1970, Bethesda, Maryland, published by the U.S. Department of Health, Education and Welfare. The classification system described below satisfies the requirements of the LRC program and is designed to allow the unambiguous assignment of patients to one of several categories if their lipoprotein profiles satisfy certain specific criteria. It is recognized that the lipoprotein profiles of some patients may satisfy some but not all of these criteria. When this is observed the patients are arbitrarily assigned to a category designated as "Other" pending further consideration when the data are analyzed at the completion of the study. For example, a patient who possesses "floating beta" lipoprotein and who satisfies other clinical criteria for Type III hyperlipoproteinemia, but whose plasma VLDL cholesterol to triglyceride ratio is less than 0.3 is classified as "Other" rather than as Type III, for purposes of the LRC program. This assignment is made even though the medical management of the patient might follow procedures appropriate to Type III hyperlipoproteinemia. The lipoprotein patterns described should not be equated with specific diseases since the interpretation of these patterns, in the conventional medical setting, includes consideration of other clinical tests, physical findings and family studies.

9.2 Definitions of the Normal Plasma Lipoproteins

The plasma lipoproteins are usually separated into four fam-

ilies. The usual nomenclature employed, their density and their ultracentrifugal and electrophoretic characteristics are indicated in Table 8. Under defined conditions the chemical composition of a given lipoprotein family is generally reproducible and it is possible to express the lipoprotein concentrations in terms of their cholesterol content.

9.3 Provisional Normal Ranges for Plasma Lipids and Lipoproteins

Provisional normal ranges for plasma, VLDL, LDL, and HDL cholesterol concentrations and the plasma triglyceride concentrations are shown in Table 7. Chylomicrons are judged to be either present or absent by the Standing Plasma Test (sec. 4.1.2.).

9.4 Normal and Abnormal Lipoprotein Profiles

The following lipoprotein patterns are recognized on the basis of the lipid and lipoprotein analyses described in this volume. The determination of whether the observed patterns are primary or secondary to other disease states is based, in the LRC program, on the results of a battery of clinical chemical tests. The following algorithms are used to establish a lipoprotein type.

9.4.1. Normal Lipoprotein Profile

A) LRC Criteria

For purposes of the LRC project a patient will be designated as "normal" if his lipoprotein profile satisfies the following criteria:

1) LDL concentration is between the age-adjusted 5th and 95th percentile (Table 9).

2) Concentration of plasma triglycerides is between the age-adjusted 5th and 95th percentile (Table 9).

3) HDL concentration is between the age and sex adjusted

lower 5th and upper 95th percentile.

4) Plasma contains no detectable "floating beta" lipoprotein by paper or agarose gel electrophoresis.

5) VLDL cholesterol: plasma triglyceride ratio is <0.3.

6) Plasma contains no chylomicrons by the Standing Plasma Test (sec. 4.1.2.).

7) Plasma may or may not contain "sinking prebeta" lipoprotein (sec. 9.4.5.).

9.4.2. Types I-V Hyperlipoproteinemia (see Fig. 8)

A) Type I (hyperchylomicronemia with lipoprotein lipase deficiency)

1) LRC Program Criteria

a) Presence of chylomicrons above a clear infranatant as determined by the Standing Plasma Test (sec. 4.1.2.) and plasma triglyceride concentration >400 mg/100 ml.

b) Deficiency of lipoprotein lipase as determined by the Intravenous Heparin Strip Test (sec. 8).

2) Other findings (not part of LRC algorithm for Type I) include normal or elevated concentrations of VLDL and normal or reduced concentrations of LDL and HDL.

B) Type IIa (Hyperbetalipoproteinemia)

1) LRC Program Criteria

a) LDL concentration above the age-adjusted upper 95th percentile (Table 9).

b) Plasma triglyceride concentration below the age-adjusted upper 95th percentile (Table 9), with absence of "floating beta" lipoprotein

(sec. 5.) and chylomicrons (sec. 4.1.2.).

C) Type IIb (hyperbeta-hyperprebetalipoproteinemia)

1) LRC Program Criteria

a) LDL concentration above the age-adjusted upper 95th percentile (Table 9).

b) Plasma triglyceride concentration above the age-adjusted upper 95th percentile, with absence of "floating beta" lipoprotein (sec. 5.) and chylomicrons (sec. 4.1.2.).

D) Type III (Dysbetalipoproteinemia)

1) LRC Program Criteria

a) Presence of lipoproteins with beta mobility on paper or agarose gel electrophoresis (sec. 5.) of the ultracentrifugal top fraction ($d < 1.006$).

b) Ratio of "VLDL cholesterol" to plasma triglycerides > 0.3 when the concentration of plasma triglycerides is 150-1000 mg/100 ml. This criterion need not be satisfied when the concentration of plasma triglycerides is less than 150 mg/100 ml or greater than 1000 mg/100 ml.

c) When the concentration of plasma triglycerides is less than 150 mg/100 ml, errors in the analysis of low triglyceride levels and of the corresponding small amounts of VLDL cholesterol may make the ratio unreliable. It is also possible that the VLDL cholesterol:plasma triglyceride ratio may be < 0.3 in some patients who, on the basis of the presence of "floating beta" lipo-

protein and other criteria do have Type III hyperlipoproteinemia. It is anticipated that the LRC studies will illuminate this question. Plasma triglyceride concentrations > 1000 mg/100 ml in Type III subjects often indicate the presence of chylomicrons which may cause the VLDL cholesterol:plasma triglyceride ratio to fall below 0.3. For LRC purposes, patients who do not satisfy both criteria a) and b) are classified as "Other".

E) Type IV (Hyperprebetalipoproteinemia)

1) LRC Program Criteria

a) Concentration of plasma triglycerides is above the age-adjusted upper 95th percentile (Table 9) in the absence of chylomicrons (sec. 4.1.2.) and "floating beta" lipoprotein.

b) LDL cholesterol concentration is below the age-adjusted upper 95th percentile.

F) Type V (Hyperchylomicronemia without lipoprotein lipase deficiency)

1) LRC Program Criteria

a) Concentration of plasma triglycerides above 400 mg/100 ml.

b) Presence of chylomicrons by the Standing Plasma Test (sec. 4.1.2.).

c) No deficiency of lipoprotein lipase as determined by the Intravenous Heparin Strip Test (sec. 8).

d) LDL concentrations below the age-adjusted upper 95th percentile (Table 9).

e) Absence of "floating beta" lipoprotein.

2) Other findings (not part of LRC algorithm for Type V) include increased concentrations of VLDL as evidenced by turbidity of the plasma infranatant by the Standing Plasma Test (sec. 4.1.2.) or by electrophoresis.

9.4.3. Additional Lipoprotein Profiles

A) Hyperalphalipoproteinemia

1) LRC Program Criteria

a) HDL concentration above the age and sex adjusted upper 95th percentile (Table 9).

b) Otherwise "normal" lipoprotein profile.

B) Abetalipoproteinemia

Plasma cholesterol concentration is not greater than 80 mg/100 ml and is usually less than 50 mg/100 ml. The concentration of plasma triglycerides is less than 20 mg/100 ml (D.S. Fredrickson, A.M. Gotto and R.I. Levy in The Metabolic Basis of Inherited Disease, 3rd Ed., J.B. Stansbury, J.B. Wyngaarden, and D.S. Fredrickson, Eds., McGraw-Hill Book Company, N.Y., Chap. 26, P. 503). Beta prebeta and chylomicron bands are absent on paper or agarose gel electrophoresis. A very small amount of lipoprotein can sometimes be measured as LDL cholesterol (about 1 mg/100 ml, measured in a single patient; Fredrickson, D.S. et al. J. Clin. Invest. 47, 2446 (1968)) but which reacts immunochemically as HDL ra-

ther than LDL. The definitive diagnosis requires the absence of immunochemically detectable LDL.

C) Hypobetalipoproteinemia

1) LRC Program Criteria

a) LDL concentration below the age-adjusted lower 95th percentile (Table 9).

D) Lipoprotein Pattern of Tangier Disease

Plasma cholesterol concentration is low and the concentration of plasma triglycerides is usually elevated. The alpha lipoprotein band is absent on paper or agarose gel electrophoresis and the concentration of HDL is less than 8 mg/100 ml. Beta migrating VLDL may also be present on paper or agarose gel electrophoresis of the ultracentrifugal top fraction ($d < 1.006$).

9.4.4. Patients to be Classified as "Other"

A) For purposes of the LRC program patients whose lipoprotein patterns are different than those specified will be classified as "Other". Some examples of "Other" classifications follow:

1) Subjects with elevated HDL and another lipoprotein abnormality.

2) Subjects possessing "floating beta" lipoprotein, plasma triglyceride concentrations between 150-1000 mg/100 ml and VLDL cholesterol to plasma triglyceride ratio < 0.3.

3) Subjects with no detectable "floating beta" lipoprotein by paper or agarose gel electrophoresis, concentration of plasma triglyceride 150-1000 mg/100 ml

and VLDL cholesterol to plasma triglyceride ratio > 0.3.

4) Subjects with chylomicrons by the Standing Plasma Test (sec. 4.1.2.) and concentrations of plasma triglycerides less than 400 mg/100 ml. These subjects are not classified Type I or Type V because this assignment also requires performance of the Intravenous Heparin Strip Test (sec. 8). (It is unlikely that a Type I subject would have concentrations of plasma triglycerides less than 400 mg/100 ml.)

9.4.5. "Sinking Prebeta" Lipoprotein

Lipoprotein species with prebeta mobility on paper or agarose gel electrophoresis of the ultracentrifugal bottom fraction ($d > 1.006$) is considered to be the Lp(a) lipoprotein. Its presence or absence appears to be unrelated to the occurrence of any of the lipoprotein types described above.

TABLE 1

DETAILS OF AUTOANALYZER I MANIFOLD
(See Figure 1)

Manifold #	Reagent Pumped	Color Code	I.D. (inches)	Type of tubing	Technicon Part #
8	water wash (TG)	blue-blue	0.065	standard	116-0532-13
9	air (TC)	yellow-yellow	0.056	standard	116-0532-12
10	$FeCl_3$ (TC)	green-green	0.073	standard	116-0532-14
11	$FeCl_3$ (TC)	purple-purple	0.081	standard	116-0532-15
12	$FeCl_3$ (TC)	purple-purple	0.081	standard	116-0532-15
13	water wash (TC)	green-green	0.073	standard	116-0532-14
14	TC sample line	yellow-yellow	0.056	solvaflex	116-0533-12
15	waste (TC)	purple-purple	0.081	standard	116-0532-15
16	waste (TC)	purple-black	0.090	standard	116-0532-16
17	periodate (TG)	grey-grey	0.051	standard	116-0532-11
18	acetylacetone (TG)	blue-blue	0.065	standard	116-0532-13
19	TG sample line	black-black	0.030	solvaflex	116-0533-07
20	air (TG)	green-green	0.073	standard	116-0532-14
21	KOH (TG)	green-green	0.073	acidflex*	116-0535-14
22	KOH (TG)	green-green	0.073	acidflex*	116-0535-14
23	waste (TG)	purple-white	0.110	solvaflex	116-0533-18
	(on line mixing, see	purple-purple	0.081	solvaflex	116-0533-15
	section 2.2.2.E.)	orange-orange	0.035	standard	116-0532-08

Notes

TG: For transmission tubing use 0.0625 (1/16" x 1/8") solvaflex.
For interconnections on heating bath use 0.090 solvaflex.

TC: To the heating bath use 0.0625 acidflex.
From the heating bath use 0.081, 12" long acidflex to the H3 fitting.

Use macroprobes for both sample pickups; cam: 40 per hour, 2:1 sample to wash ratio

TG: Part no. 116-0152-01 Single, 7 turn, mixing coil
Part no. 116-0152-02 Double, 14 turn, mixing coil

TC: Part no. 116-0148 Jacketed, Vacuum, single mixing coil.
Fluorometer: Part no. 518-7023 Primary filter (interference) 400 nm
Part n. 518-7005 Secondary filter (sharp cut) 485 nm

*These tubes may be replaced as specified in section 2.2.2.E.

TABLE 2

ARRANGEMENT OF ISOPROPANOL BLANKS, STANDARDS, CONTROL POOLS AND SAMPLES ON TRAYS OF AUTOANALYZER I

	1	2	3	4	5	6	7	8	9	10	11	12-40
Tray 1	IPA	IPA	IPA	S_1	S_2	S_3	S_4	S_4	S_3	S_2	S_1	These cups are available for samples, quality control pools (see Table 3)
Tray 2	IPA	IPA	IPA	S_1	S_2	S_3	S_4	S_4	S_3	S_2	S_1	
	IPA	IPA	IPA	S_1	S_2	S_3	S_4	S_4	S_3	S_2	S_1	
etc.												
Postrun	IPA	IPA	IPA	S_1	S_2	S_3	S_4	S_4	S_3	S_2	S_1	

Notes

IPA refers to a wash cup of 95% zeolite-treated isopropanol. S_1, S_2, S_3, S_4 refer to zeolite-treated standards prepared as described in section 2.1.5.

The placement of four normal and four high control pools within tray positions 12-40 is shown in Table 3. Note that this number of control pools is always included in a day's run, regardless of the total number of trays run that day.

TABLE 3

ARRANGEMENT OF FOUR LOW AND FOUR HIGH SERUM QUALITY CONTROL POOLS ON AAI TRAYS
DURING RUNS OF LENGTHS VARYING FROM ONE TO EIGHT TRAYS
(Figures given refer to cup # on a particular tray)

Type of Control	Tray# 1	2	3	4	5	6	7	8
Low	15 21 28 34							
High	18 24 31 37							
Low	18 29	23 34						
High	23 34	18 29						
Low	19 33	26	21					
High	26	19 33	31					
Low	21	31	21	31				
High	31	21	31	21				
Low	21	26	31	--	21			
High	31	--	21	26	31			
Low	21	26	--	26	--	21		
High	31	--	26	--	26	31		
Low	26	--	26	--	26	--	21	
High	--	26	--	26	--	26	31	
Low	26	--	26	--	26	--	26	--
High	--	26	--	26	--	26	--	26

T3

TABLE 4

TRANSMISSION TUBING SIZES SUGGESTED FOR DETERMINATION OF CHOLESTEROL AND TRIGLYCERIDES ON THE AAII

Tubing	Location	Size, ID (inches)	Type
Cholesterol wash (H_2O)	Pump to Sampler IV	0.073	Tygon
Triglyceride wash (60% IPA)	Pump to Sampler IV	0.065	Solvaflex
LB line	Pump to mixing coil (Before sample introduction)	0.073	Tygon
80% Isopropanol	Pump to airgun	0.051	Solvaflex
KOH	Pump to airgun	0.020	Tygon
Periodate reagent	Pump to introduction fitting between heating baths	0.045	Tygon
Acetylacetone reagent	Pump to introduction fitting between heating baths	0.051	Tygon
LB reagent pull-through line	Pump to waste	0.065	Tygon
Triglyceride pull-through lines	Pump to waste	0.056	Solvaflex

Notes

Transmission tubing from pump to cartridges should be no longer than 15" for triglyceride and 8" for cholesterol.

TABLE 5

ARRANGEMENT OF ISOPROPANOL BLANKS, STANDARDS, CONTROL POOLS, CALIBRATORS, AND SAMPLES ON TRAYS OF AUTOANALYZER II

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16-39	40
Tray 1	IPA	IPA	IPA	S_3	S_3	IPA	S_1	S_2	S_3	S_4	S_3	Ser-cal	Ser-cal	S_3	S_3	Samples and quality control	IPA
Tray 2	IPA	IPA	IPA	S_3	S_3	These cups are available for samples and quality control pools.											IPA

Each succeeding tray of the run has the same arrangement of isopropanol blanks, standards, control pools, calibrators, and samples.

Notes

IPA refers to a wash cup containing 95% Zeolite-treated isopropanol. S_1, S_2, S_3, S_4 refer to Zeolite-treated standards prepared as described in section 2.1.5.

The placement of four low and four high control pools within the proper tray positions is shown in Table 6. Note that this number of control pools is always included in a day's run, regardless of the total number of trays.

TABLE 6

ARRANGEMENT OF FOUR LOW AND FOUR HIGH CONTROL POOLS ON AAII TRAYS
DURING RUNS OF LENGTHS VARYING FROM ONE TO EIGHT TRAYS
(Figures given refer to cup # on a particular tray)

Type of Control	Tray # 1	2	3	4	5	6	7	8
High	16 21 28 34							
Low	18 24 31 34							
High	20 35	14 29						
Low	28 --	7 22 37						
High	25	13 37	25					
Low	37	25	13 37					
High	29	26	22	15				
Low	--	10	16	39 32				
High	34	--	6	11	18			
Low	--	19	26	39	39			
High	36	--	18	33	--	39		
Low	--	27	--	9	24	15		
High	26	--	18	--	10	35	--	
Low	--	22	--	14	39	--	31	
High	26	--	26	--	26	--	26	--
Low	--	26	--	26	--	26	--	26

TABLE 7

CDC STANDARDIZATION CRITERIA FOR ACCURACY AND PRECISION FOR VARIOUS CHOLESTEROL AND TRIGLYCERIDE CONCENTRATIONS

Cholesterol concentration (mg/100 ml)	Deviation from target value (T.V.) T.V.± 5% of T.V. (mg/100 ml)	Overall standard deviation* (mg/100 ml)
100-199	5.00- 9.95	< 7.00
200-299	10.00-14.95	< 8.00
300-399	15.00-19.95	< 9.00
400-499	20.00-24.95	<10.00

Triglyceride concentration (mg/100 ml)	Deviation from target value (mg/100 ml)	Overall standard deviation*
0- 74	± 9	< 7.00
75-149	± 9	< 8.00
150-199	± 9	< 9.00
200-249	± 9	<10.00
250-299	± 13	<12.00
300-349	± 13	<14.00
350-399	± 15	<16.00
400-499	± 17	<18.00

*Overall standard deviation is the total of the within day and day-to-day components of variance derived by the analysis of variance technique.

TABLE 8

MAJOR LIPOPROTEIN FAMILIES

Family Name	Synonyms	Electrophoretic Definition[a]	Ultracentrifugal Definition[b]
Chylomicrons	--	Remain at origin[c]	$D < 0.95$, $S_f > 400$
Very low density lipoproteins (VLDL)	Prebeta lipoproteins	Prebeta-mobility	$0.95 \leq D < 1.006$, $20 < S_f \leq 400$
Low density lipoproteins (LDL)	Beta lipoproteins	Beta-mobility	$1.006 \leq D < 1.063$[d], $0 < S_f \leq 20$
High density lipoproteins (HDL)	Alpha lipoproteins	$Alpha_1$-mobility	$1.063 \leq D < 1.21$[e]

[a] On paper electrophoresis

[b] Expressed as densities in g/ml and in Svedberg units, S_f

[c] On starch block electrophoresis have $alpha_2$ mobility

[d] LDL includes the sub-classes of $1.006 \leq D < 1.019$ ($12 \leq S_f < 20$) and of $1.019 \leq D < 1.063$ ($0 \leq S_f < 12$)

[e] HDL includes the sub-classes of HDL_2 ($1.063 \leq D < 1.12$) and HDL_3 ($1.12 \leq D < 1.21$)

TABLE 9
NORMAL DISTRIBUTIONS* FOR
PLASMA LIPIDS AND LIPOPROTEIN CHOLESTEROL**

Age	Total Plasma# Cholesterol mg/100 ml	Plasma Triglyceride mg/100 ml	VLDL Cholesterol mg/100 ml	LDL Cholesterol mg/100 ml	HDL Cholesterol mg/100 ml	
					Male	Female
0-19	120-230	10-140	5-25	50-170	30-65	30-70
20-29	120-240	10-140	5-25	60-170	35-70	35-75
30-39	140-270	10-150	5-35	70-190	30-65	35-80
40-49	150-310	10-160	5-35	80-190	30-65	40-95
50-59	160-330	10-190	10-40	80-210	30-65	35-85

* Based on 90% fiducial limits calculated for small samples. All values are rounded to the nearest 5 mg/100 ml and for practical purposes differences between sexes have been ignored except for HDL cholesterol concentrations.

** From Section on Lipoproteins, Laboratory for Molecular Diseases, National Heart and Lung Institute, NIH, Bethesda, Maryland.

Not used by LRC program but included here for convenience. A small but significant proportion of Type II patients have total cholesterol between the 90th and 95th percentile.

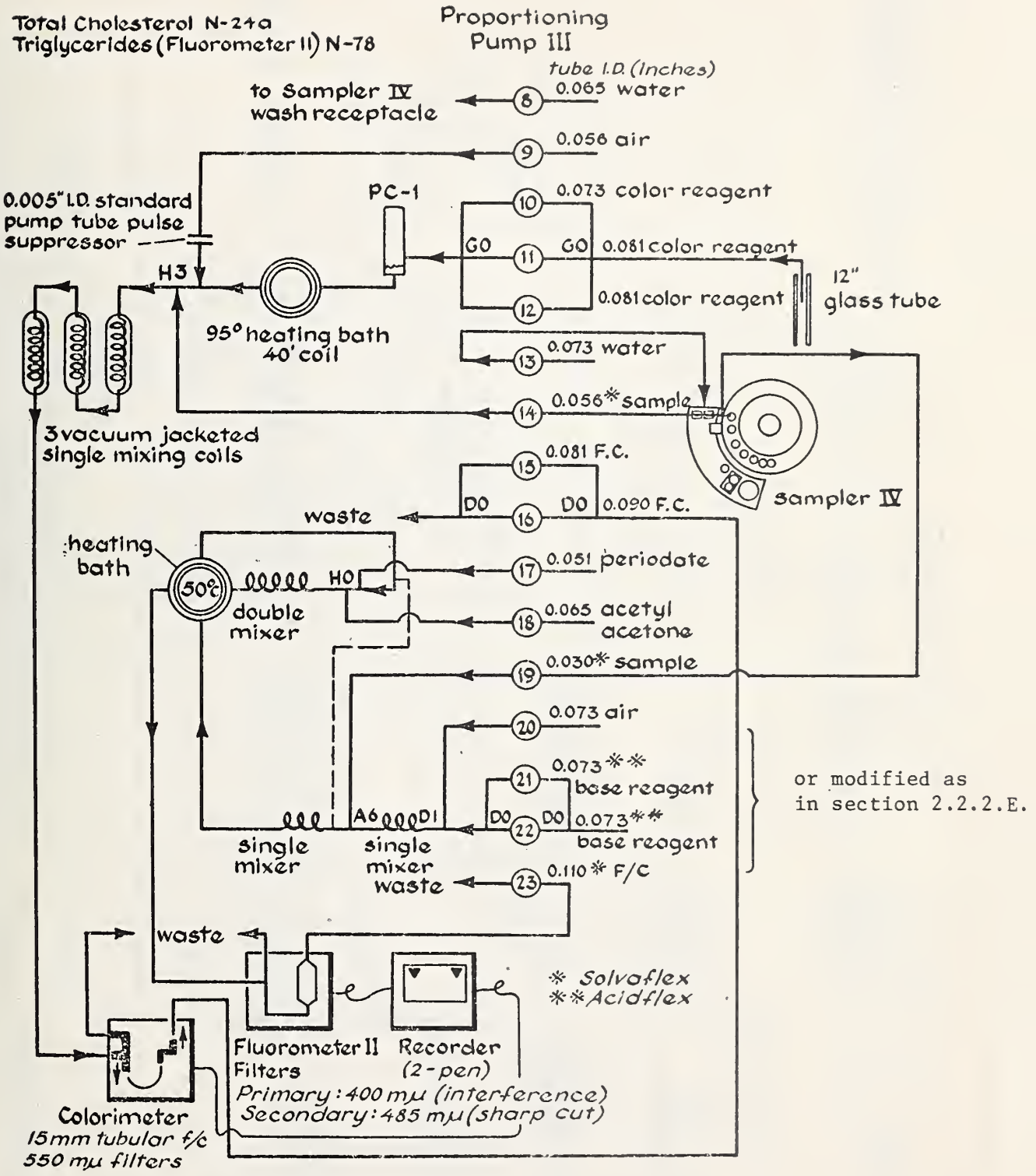


Fig. 1. Flow Sheet for Simultaneous Determination of Cholesterol and Triglyceride Concentrations in Plasma by AutoAnalyzer I

----indicates bypass path for TG blanks

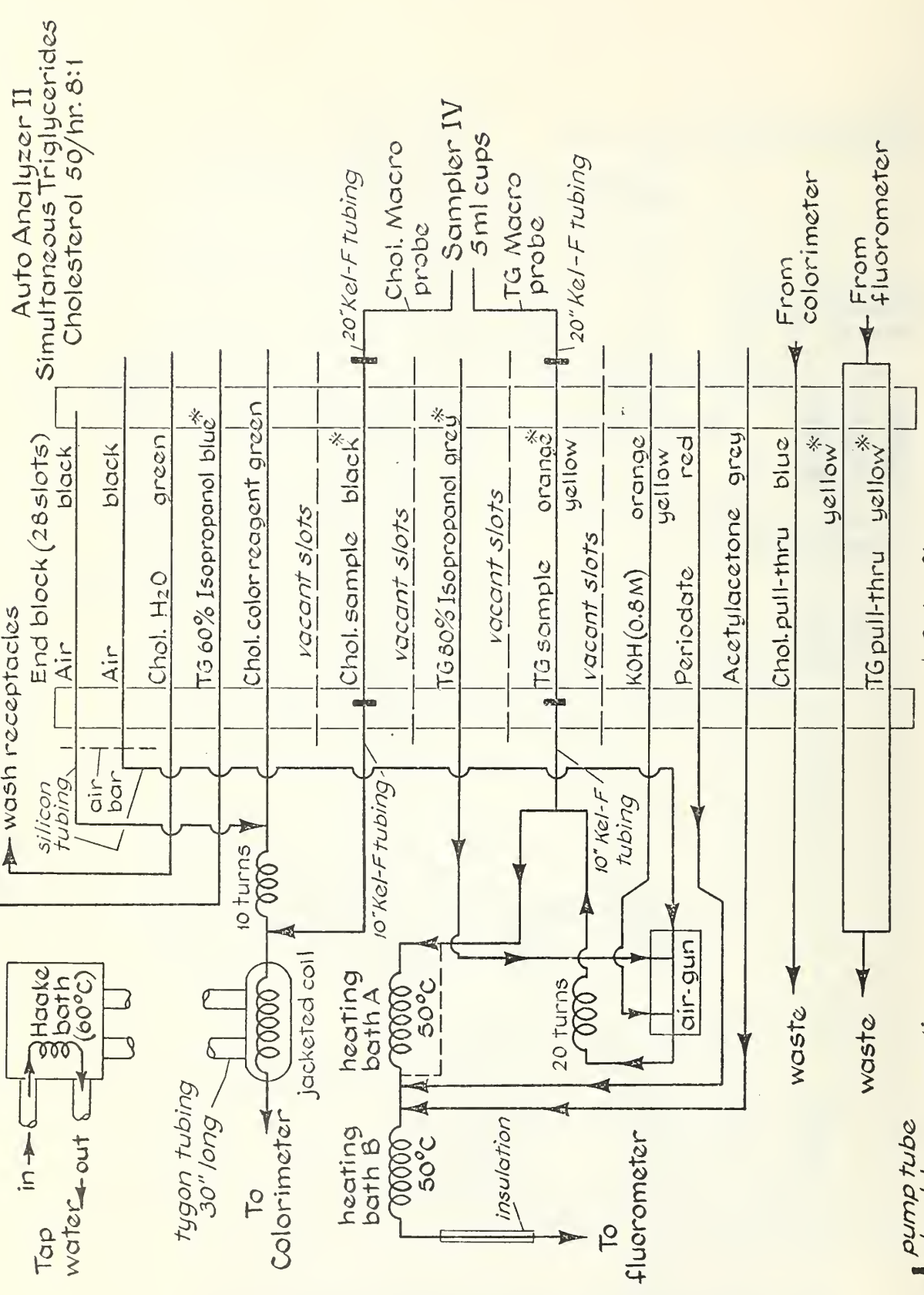
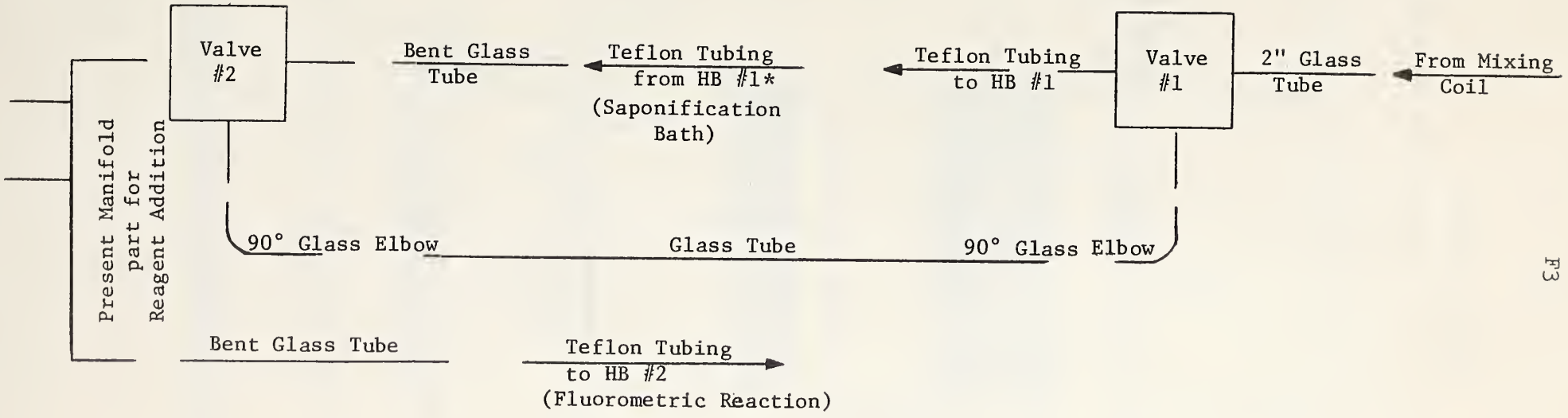


Fig. 2. Flow Sheet for Simultaneous Determination of Total Cholesterol and Triglyceride Concentrations in Plasma by AutoAnalyzer II

----indicates bypass path for TG blanks



*HB = Heating Bath.

Fig. 3. Triglyceride Cartridge Modification--AAII

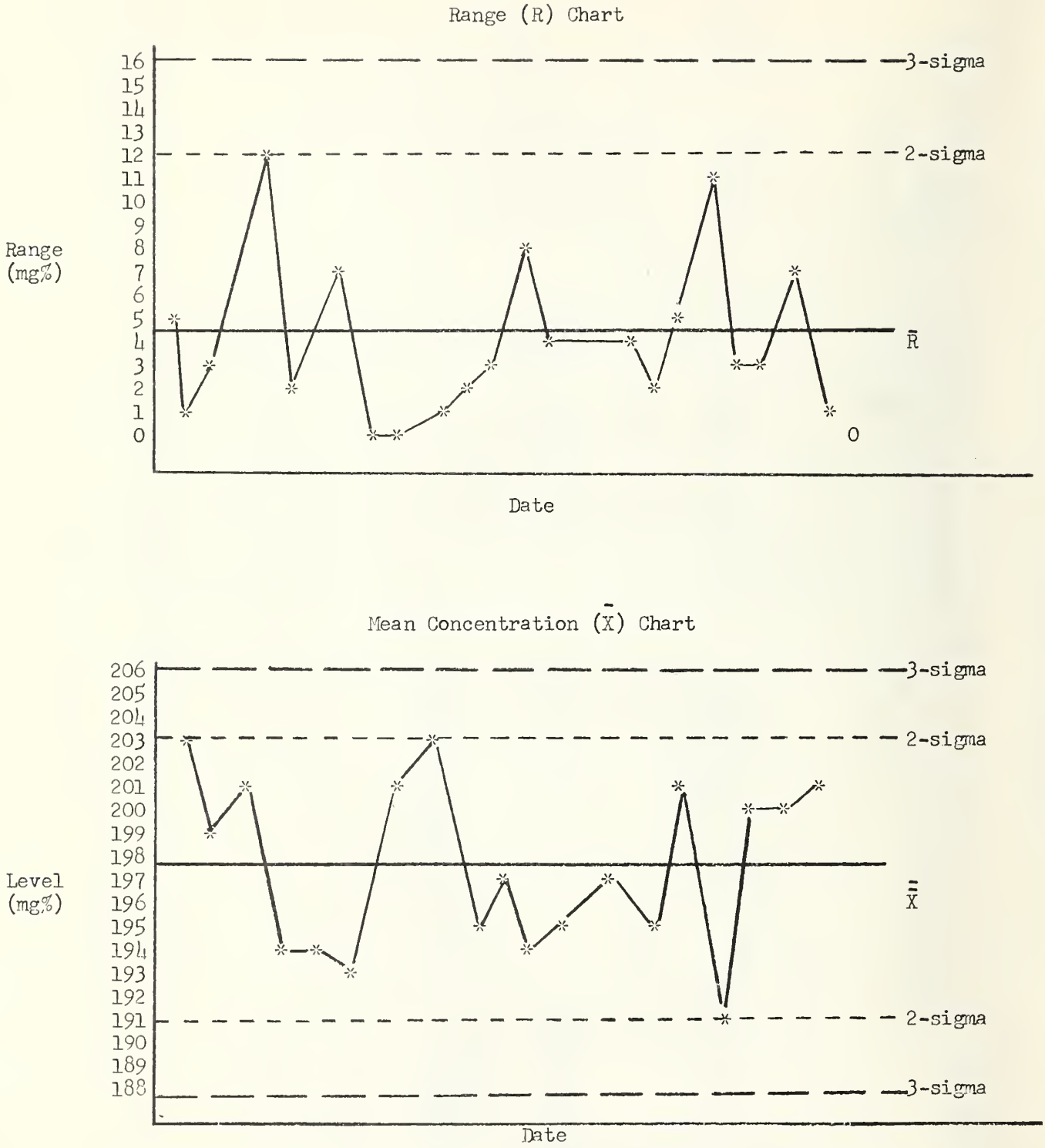


Fig. 4. Examples of Range and Mean Quality Control Charts

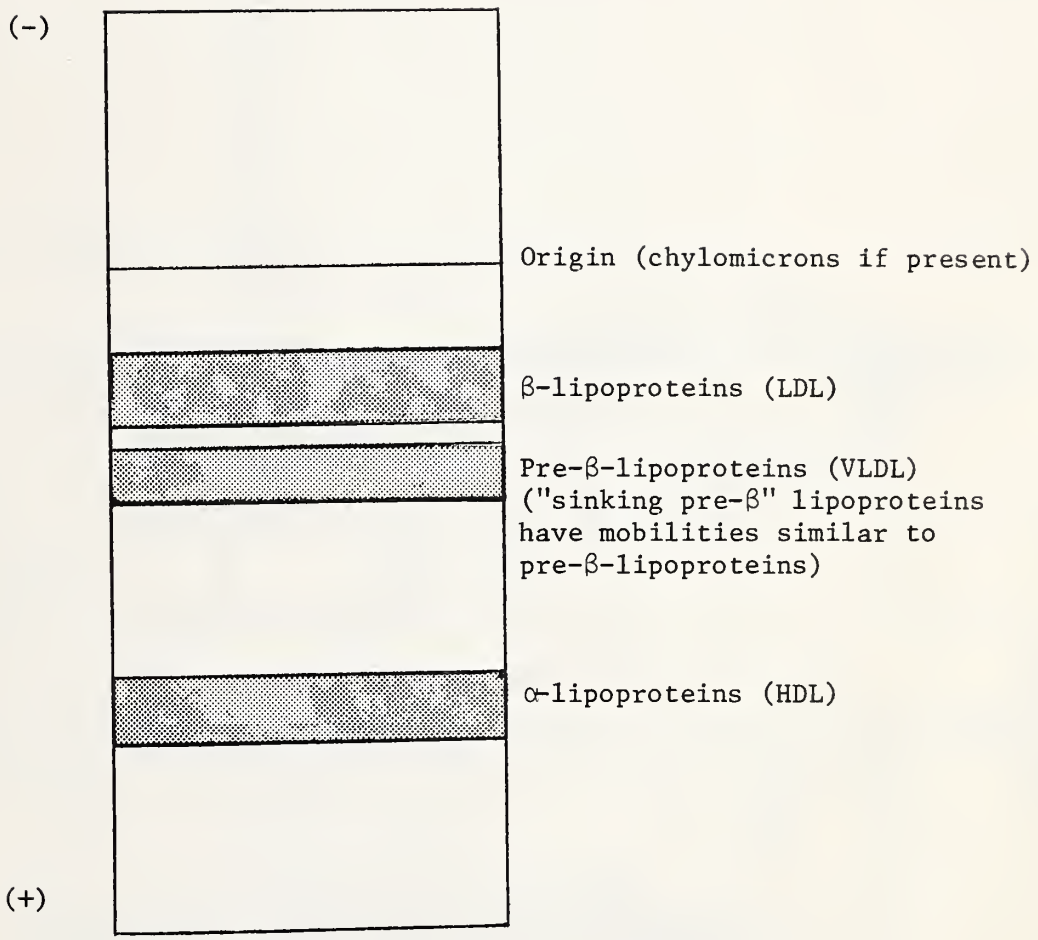


Fig. 5. Migration of Normal Lipoproteins on Paper or Agarose Electrophoresis

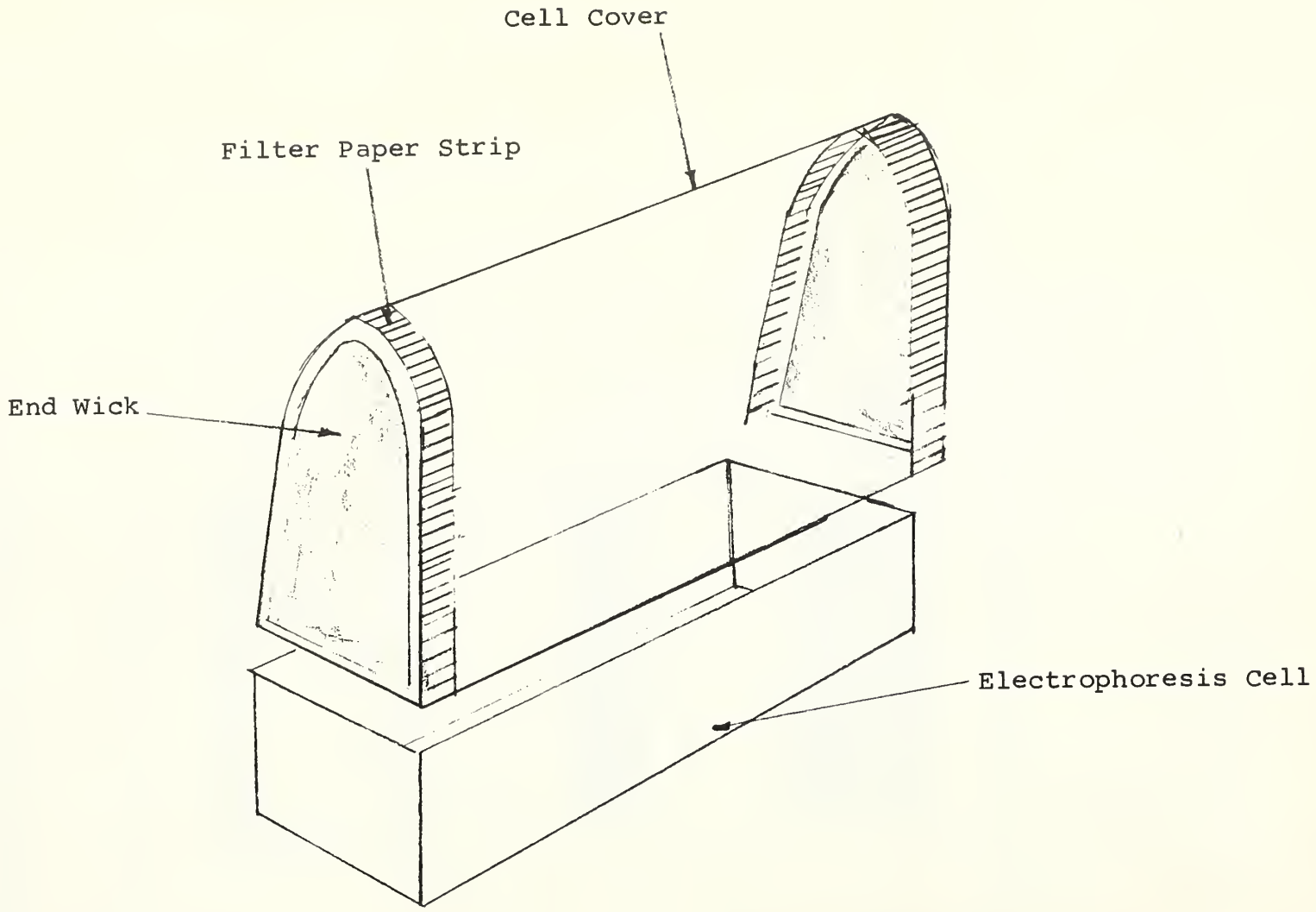


Fig. 6. Durrum Type Cell for Paper Electrophoresis

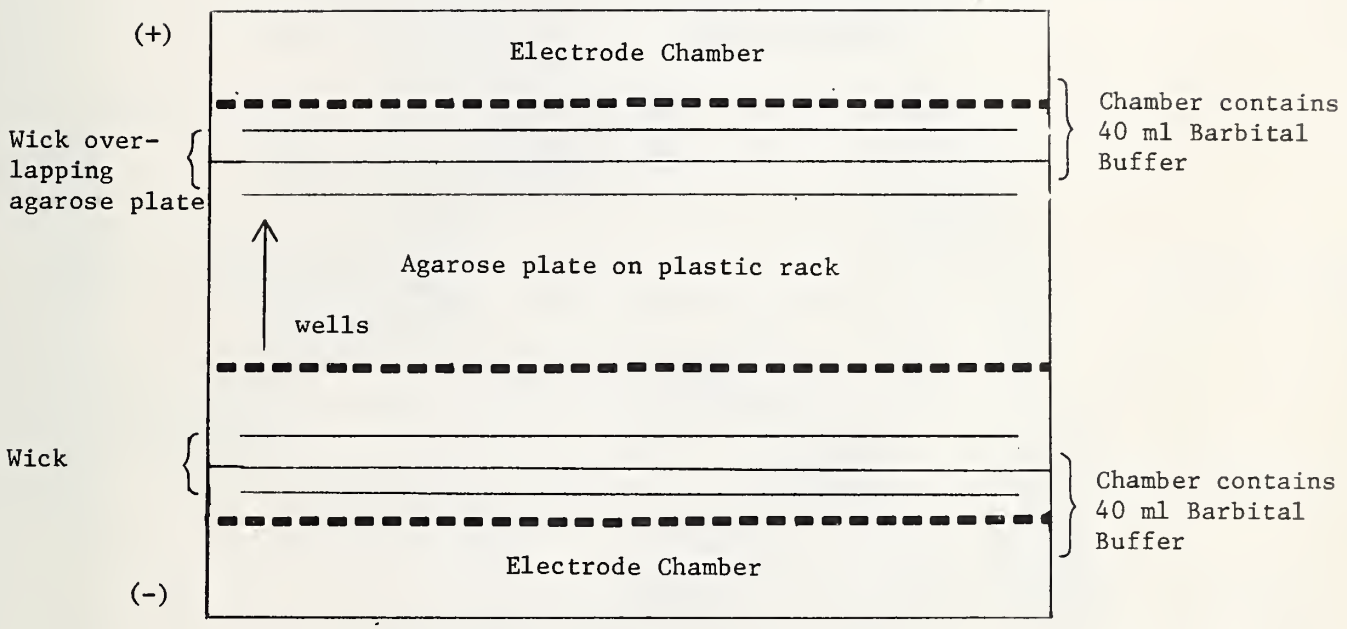


Fig. 7 Electrophoresis Cell for Agarose Electrophoresis of Plasma Lipoproteins

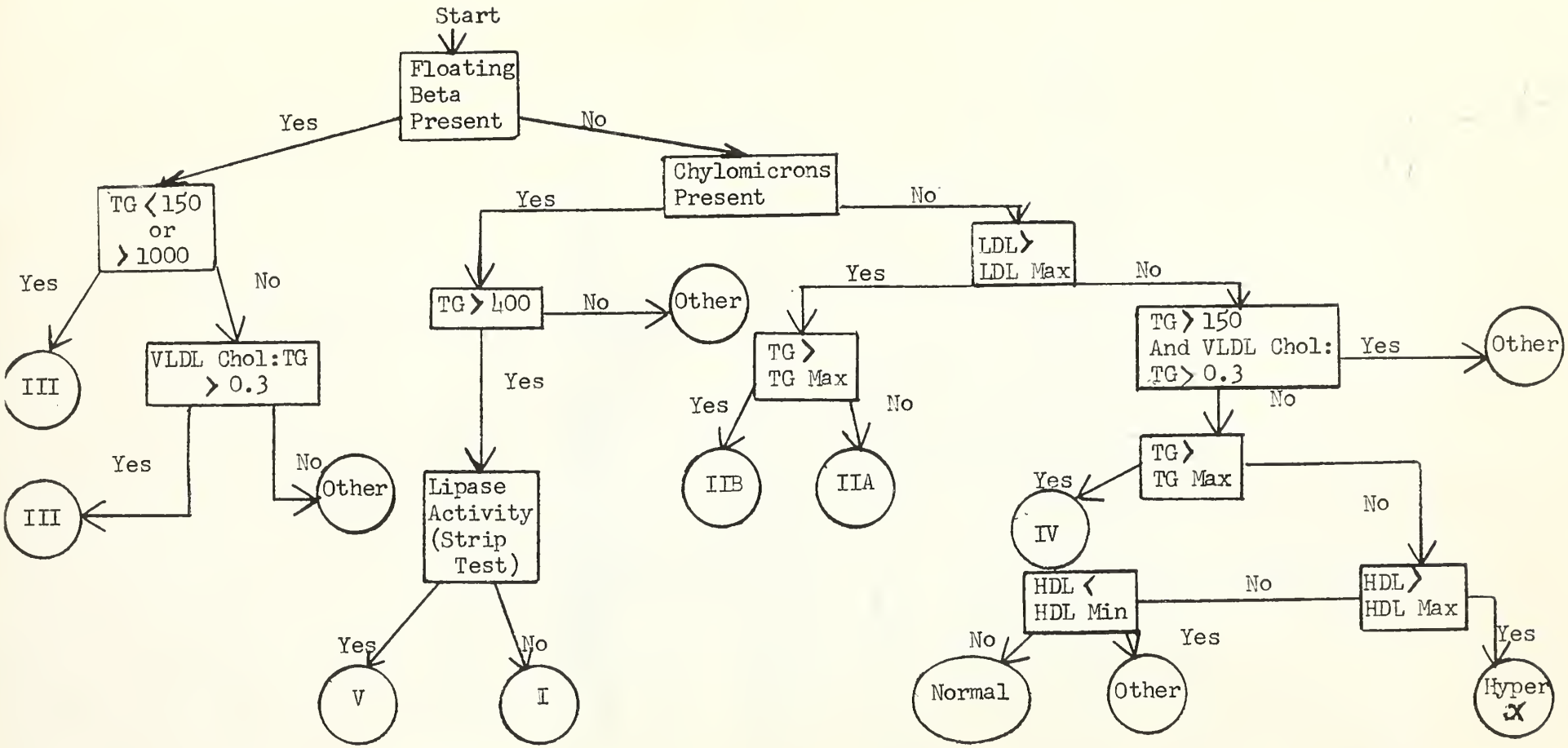


Figure 8 - Flowchart For Typing System

Appendix I

Sources of Supplies and Equipment

A. Blood Drawing Supplies

1. Tourniquet
   Velket™ Tourniquet, adult size
   Velcro Manufacturing Co., Long Island, N.Y.

2. Blood drawing tubes (specify silicone-lubricated stoppers)
   Vacutainer™ evacuated glass tube, 15 ml, containing 16 mg solid EDTA (purple stopper)
   Cat. No. 3218XF282
   Becton Dickenson and Co., Rutherford, N.J.

3. Needles
   20 g 1-1/2" non-drip multiple sample needle
   Cat. No. 5749
   Becton Dickenson and Co., Rutherford, N.J.

4. Antiseptic Swab
   Antispetic swab containing benzalkanium chloride in isopropanol (1:750)
   Cat. No. 3500
   MedPak Corp., Charleston, W. Va.

5. Pediatric blood drawing equipment

   a. Butterfly-23 Infusion Set, 23 G thin wall needle (22 G bore) 20 sets/box
      Cat. No. 4565

   b. Butterfly Short-25 Pediatric Infusion Set, 3/8" 25-G Thinwall needle (24 G bore) 20 sets/box
      Cat. No. 4573
      Abbot Laboratories, Abbott Park, No. Chicago, Ill.

6. Gauze pad
   Gauze pad, 2" x 2", sterile, dry
   Cat. No. 20-629-13
   Parke-Davis and Co., Detroit, Mich.

B. Sample storage vials

20 ml Liquid Scintillation Vials with polyseal cone cap liner. 500/case
Cat. No. 6001050
Packard Instrument Co., 2200 Warrenville Rd., Daniel Grove, Ill.

C. Materials for shipment of unfrozen samples

1. Shipping container
   Insulated shipping container
   Cat. No. 38-PW
   Plyfoam Packers Co., 6415 California Ave., Chicago, Ill.

2. Plastic bag
   Sears Poultry Food Bag, 8" x 4" x 16"
   Sears Roebuck and Company

3. Styrofoam sheet holders
   Perforated styrofoam sheets
   Pycofoam Corp., P. O. Box 187, Norristown, Pa.

D. Materials for preparation of sample extracts

1. Automatic pipet
   MicroMedic Automatic Pipet
   Model No. 25000 ATF
   Micromedic Systems, Inc., Rohm & Haas Bldg., Philadelphia, Pa.

2. Sample extraction tubes and caps
   Test tube 16 x 125 mm, screw cap, disposable
   Cat. No. 99447
   Teflon™-lined caps
   Cat. No. 9998
   Corning Glassware, Corning, N.Y.

3. Refrigerated centrifuge
   Refrigerated centrifuge
   Model No. PR-2
   International Equipment Co., 300 Second Ave., Needham Heights, Mass.

E. Reagents for AutoAnalyzer™I

1. Isopropanol
   Isopropanol, Mallinkrodt, Spectro Grade
   Mallinkrodt Chemical Works, St. Louis, Mo.

2. Zeolite mixture
   Zeolite mixture, anhydrous, 200g
   Cat. No. T11-0375
   Technicon Instrument Corp., Tarrytown, N.Y.

3. Potassium hydroxide in isopropanol
   Base reagent 1L
   Cat. No. T01-0369
   Technicon Instruments Corp., Tarrytown, N.Y.

4. Sodium periodate solution
   Sodium periodate reagent, 1L
   Cat. No. T01-0372
   Technicon Instruments Corp., Tarrytown, N.Y.

5. Ammonium acetate solution
   Ammonium acetate, 2M, 1L
   Cat. No. T21-0373
   Technicon Instruments Corp., Tarrytown, N.Y.

6. Acetylacetone
   Acetylacetone, 100 ml
   Cat. No. T01-0379
   Technicon Instruments Corp., Tarrytown, N.Y.

F. Reagents for AutoAnalyzer™ II

1. Potassium hydroxide solution
   Potassium Hydroxide, 0.8M, 1L
   Cat. No. T01-0473
   Technicon Instruments Corp., Tarrytown, N.Y.

2. Sodium periodate solution
   Same as above

3. Ammonium acetate solution
   Same as above

4. Acetylacetone
   Same as above

5. Zeolite mixture
   Same as above

G. Stopcocks for TG blanking

1. AutoAnalyzer™ I
   Tomac three way stopcock
   Cat. No. 17108-010 K-75
   American Hospital Supply Co., 202 Great Southwest Pkwy., Grand Prairie, Tex.

2. AutoAnalyzer™ II
   3 way Kel-F valve
   Cat. No. 3MMM3
   Hamilton Co., P.O. Box 7500, Reno, Nev.

H. Materials for beta quantification

1. Tube slicer
   Tube slicer with 3 sets of rubber gaskets for tube sizes 1/2", 5/8" and 1" in diameter
   Cat. No. TUBS-200
   Nuclear Supply and Service Co., 422 Washington Bldg., 15th Street and New York Ave., N.W., Washington, D.C.

I. Materials for HDL cholesterol determination

1. Heparin
   L po Hepin™ Sodium Heparin, Injection
   Cat. No. NDC 89-442-01
   Riker Laboratories, Inc., Northridge, Calif.

J. Materials for laboratory-prepared agarose electrophoresis system

1. Wick, 9" x 1-1/2" cut from
   Filter paper, S & S grade 470, 23" x 23"
   Available from Chemical Supply Catalogues

2. Electrophoretic plates

Cronarfilm™, Dupont Graphic Arts Film, clear base
0.004" thick, 16" x 20"
Cat. No. C42
Riseman Graphic Arts Supply Co., 8125 Brentwood Industrial Drive,
St. Louis, Mo.

3. Nipples
Glass connector
Cat. No. N13
Technicon Instruments Corp., Tarrytown, N.Y.

4. Stain
Fat Red 7B
Robo z Surgical Instrument Co., Inc., 810 - 18th Street, N.W.
Washington, D.C.

5. Tracking Dye
Bromophenol Blue
Cat. No. B-392
Fisher Scientific Co., 711 Forbes Ave., Pittsburgh, Pa.

6. Agarose
Agarose
Seakem Marine Colloids, Inc.
Biomedical Systems, Springfield, N.J.

K. Materials for commercially prepared agarose electrophoresis system. Includes slides, sample applicator, applicator guide, electrophoresis cell and power supply unit, staining dishes and reagents.
Biogram™-A Lipoprotein Profile System
Biorad Laboratories, Clinical Div., 32nd & Griffin Ave., Richmond, Calif.

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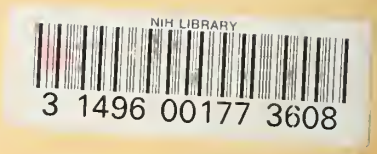

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